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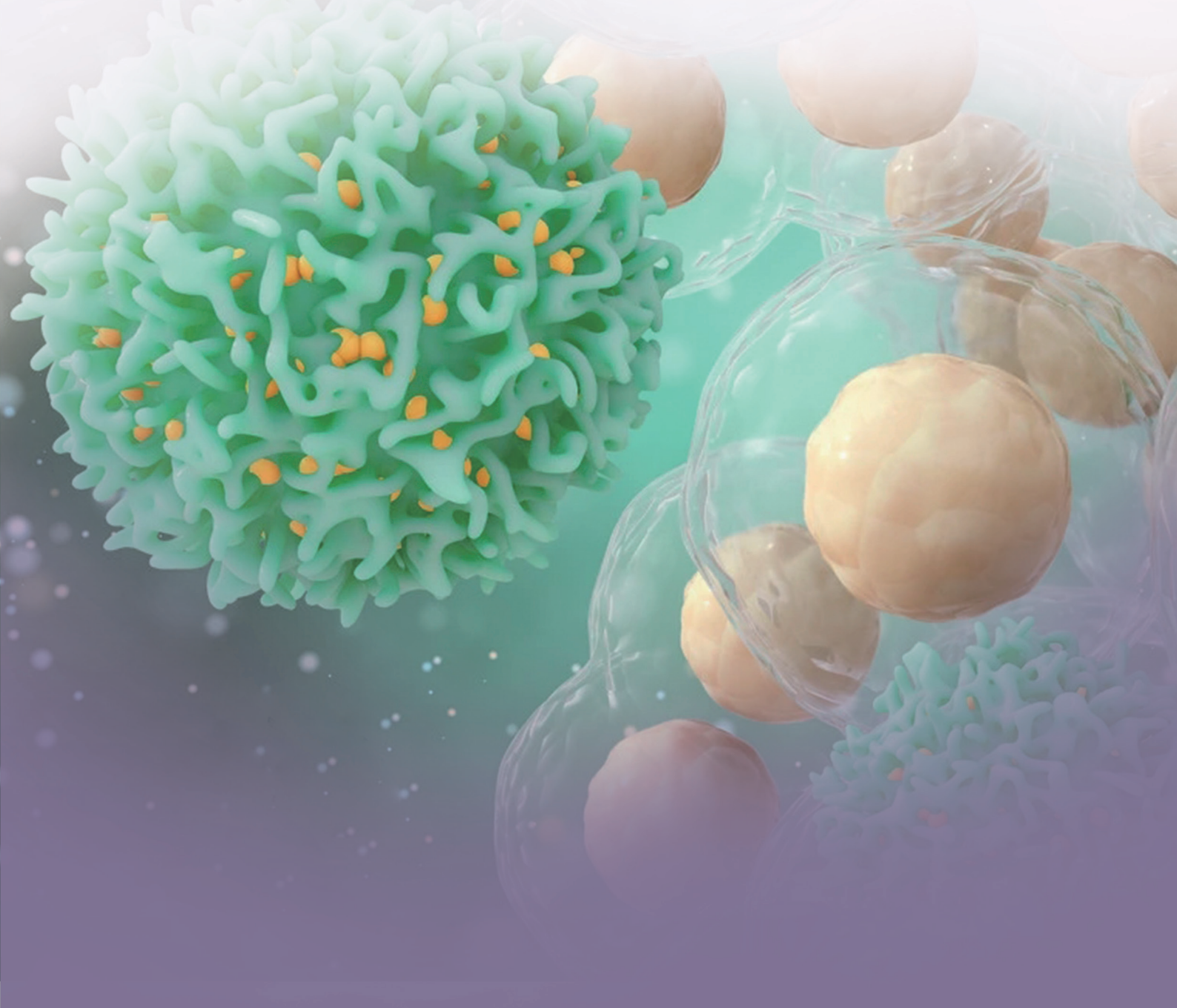

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ARTICLE

Postoperative Radiotherapy and N2 Non-small Cell Lung Cancer Prognosis: A Retrospective Study Based on Surveillance, Epidemiology, and End Results Database

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ABSTRACT

The purpose of this study is to clarify the significance of postoperative radiotherapy for N2 lung cancer. This study aimed to investigate the effect of postoperative radiotherapy on the survival and prognosis of patients with N2 lung cancer. Data from 12,000 patients with N2 lung cancer were extracted from the Surveillance, Epidemiology, and End Results database (2004-2012). Age at disease onset and 5-year survival rates were calculated. Survival curves were plotted using the Kaplan-Meier method. The univariate log-rank test was performed. Multivariate Cox regression were used to examine factors affecting survival. Patients' median age was 67 years (mean 66.46 ± 10.03). The 5-year survival rate was 12.55%. Univariate analysis revealed age, sex, pathology, and treatment regimen as factors affecting prognosis. In multivariate analysis, when compared to postoperative chemotherapy, postoperative chemoradiotherapy was better associated with survival benefits (hazard ratio [HR]= 0.85, 95% confidence interval [CI]: 0.813-0.898, P <0.001). Propensity score matching revealed that patients who had received postoperative chemoradiotherapy had a better prognosis than did patients who had received postoperative chemotherapy (HR=0.869, 95% CI: 0.817-0.925, P <0.001). Female patients and patients aged <65 years had a better prognosis than did their counterparts. Patients with adenocarcinoma had a better prognosis than did patients with squamous cell carcinoma. Moreover, prognosis worsened with increasing disease T stage. Patients who had received postoperative chemoradiotherapy had a better prognosis than did patients who had received postoperative chemotherapy. Postoperative radiotherapy was an independent prognostic factor in this patient group.

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1. Introduction

Lung cancer is a common cancer type and the leading cause of cancer-related deaths worldwide [1]. In 2018, there were 2.1 million new lung cancer cases and 1.8 million lung cancer-related deaths, accounting for 18.4% of all cancer-related deaths [2]. Non-small cell lung cancer (NSCLC) accounts for about 85% of lung cancer cases [3], among which adenocarcinoma and squamous cell carcinoma are the predominant types. Surgery is the standard treatment for NSCLC. Currently there are no guidelines on adjuvant treatment for patients with postoperative pathological N2 disease stage. Adjuvant chemotherapy is the standard treatment for patients with positive lymph node metastasis after operation, but there was a significant difference in whether adjuvant radiotherapy was performed.

2. Methods

The Surveillance, Epidemiology, and End Results (SEER) database was examined for data from lung cancer patients with a pathological diagnosis of NSCLC confirmed during 2004-2012. (Figure 1). Patients were included in the present study if they met the following criteria: (1) Their postoperative stage was N2M0; (2) Squamous cell carcinoma or adenocarcinoma was confirmed by pathology testing; (3) Postoperative treatment involved chemotherapy alone; (4) Diagnosis was confirmed during 2004-2012.

Patients were excluded from the present study if they met any of the following criteria: (1) Unclear pathology results or confirmed diagnosis of a cancer type other than squamous cell carcinoma or adenocarcinoma; (2) Confirmed metastasis; (3) Intraoperative or preoperative treatment with radiotherapy; (4) No postoperative chemotherapy; (5) Confirmed multiple primary tumors; (6) Incomplete data.

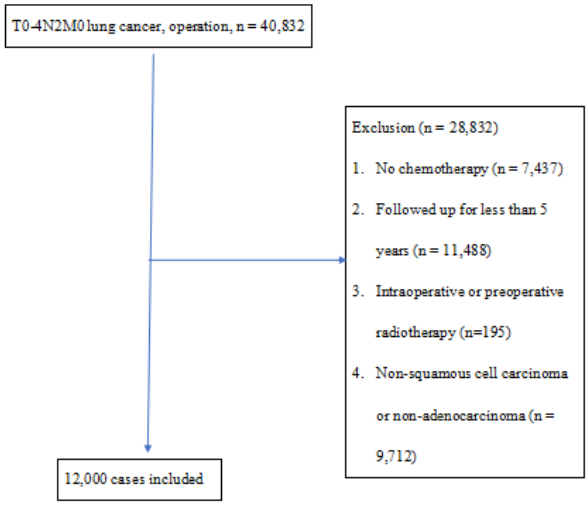

Figure 1. schematic illustration

Statistical analysis

IBM SPSS statistics version 25 was used for statistical analysis. Chi-square test was used to analyze categorical variables. Survival curves were plotted using the Kaplan-Meier method, and log-rank test was performed to analyze the differences in variables among groups. Differential variables were subjected to propensity score matching (PSM) in the postoperative chemoradiotherapy and postoperative chemotherapy group, and differences among groups were examined after matching. Univariate analysis was applied to compute models that included sex, stage, pathology, age, and treatment regimen. The variables that were significant in univariate analysis were included in multivariate analysis. Cox proportional hazards model was used for multivariate analysis. P-values <0.05 were considered statistically significant.

3. Results

Descriptive statistics

Among the 12,000 included patients. The age range was 19-95 years, with a median of 67 years (mean 66.46 ± 10.03 years). There 6127 cases (51.06%) were T0-2, 5873 cases (48.94%) were T3-4 (Table 1).

Table 1. Demographic and clinical characteristics of patients with squamous cell carcinoma and adenocarcinoma included in the Surveillance, Epidemiology, and End Results (SEER) database

Group	Cases	Percentage (%)
Sex		
Male	7119	59.33
Female	4881	40.67
Stage		
T0	66	0.55
T1	1640	13.67
T2	4421	36.84
T3	1842	15.35
T4	4031	33.59
Pathology		
Squamous cell carcinoma	6652	55.43
Adenocarcinoma	5348	44.57
Age		
≤ 65 years	5344	44.53
> 65 years	6656	55.47
Treatment		
Postoperative chemoradiotherapy	2606	21.72
Postoperative chemotherapy	9394	78.28

Univariate analysis

Kaplan-Meier curves were plotted according to sex,

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stage, pathology, age, and treatment regimen, revealing differences between groups. Women had a better prognosis than did men; patients aged 65 years and younger had a better prognosis than did patients older than 65 years; patients with adenocarcinoma had a better prognosis than did patients with squamous cell carcinoma; however, the prognosis worsened with increasing T stage. The prognosis of patients who had received postoperative chemoradiotherapy was better than that of patients who had received postoperative chemotherapy. There were statistically significant differences in gender, stage, pathology, age and treatment plan between groups ($P < 0.01$) (Figure 2).

Multivariate analyses

Multivariate analyses are carried out on the variables with significance of univariate analysis. Gender, T stage, pathology, age and treatment were included in the multivariate analysis. T stage was divided intoTt0-2 group and T3-4 group ,as shown in Table 2.

Table 2. Univariate and multivariate analyses

Group	Univariate analysis			Multivariate analysis		
	P	OR	95% CI	*P*	HR	95% CI
Sex	< 0.001	1.434	1.236-1.663	< 0.001	1.131	1.085-1.178
Stage	< 0.001	0.722	0.654-0.796	< 0.001	0.820	0.788-0.854
Pathology	< 0.001	1.348	1.158-1.569	< 0.001	1.142	1.085-1.178
Age	< 0.001	0.808	0.697-0.933	< 0.001	0.887	0.852-0.923
Treatment	< 0.001	0.807	0.697-0.934	< 0.001	0.855	0.813-0.898

HR=hazard ratio

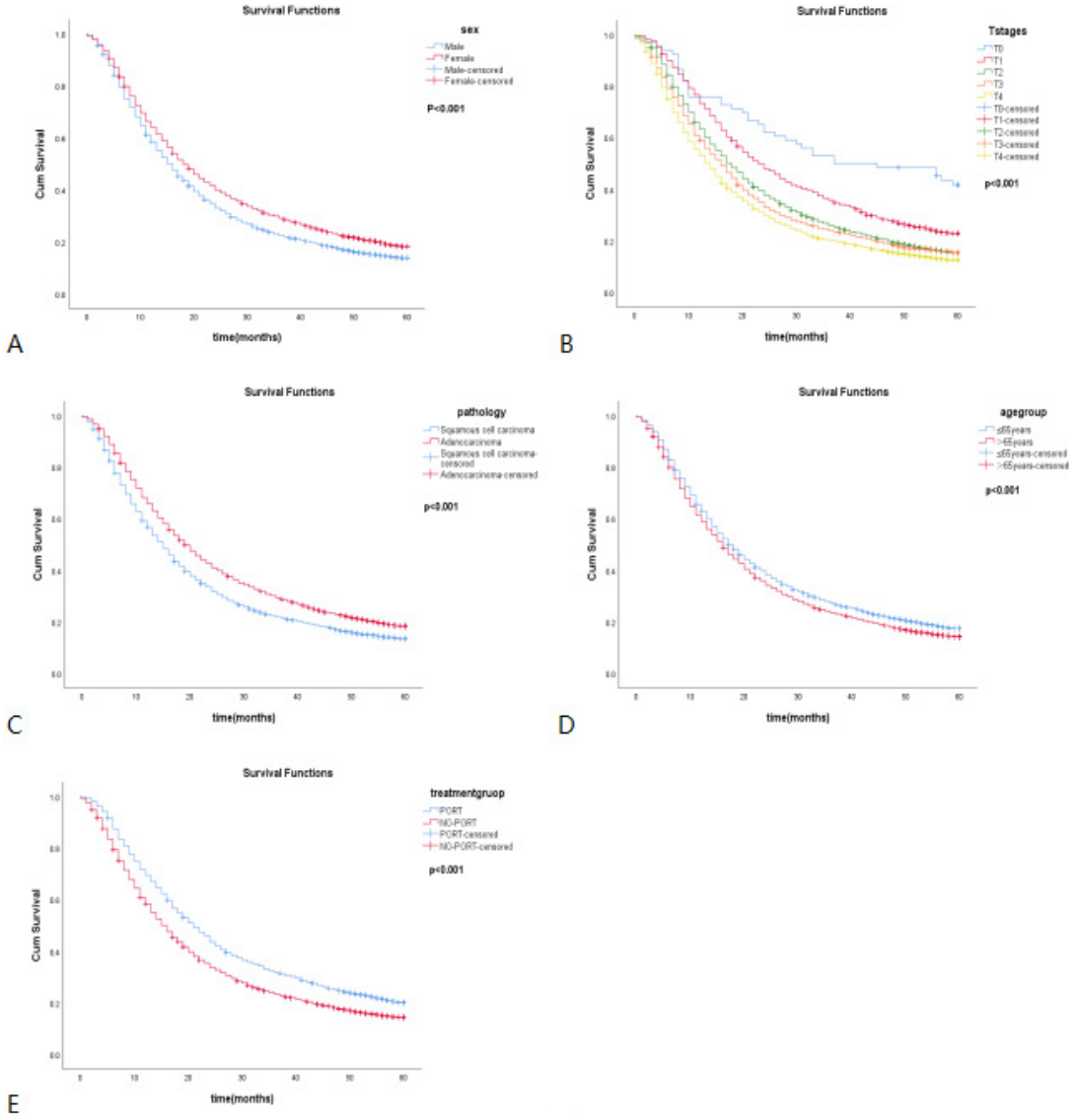

Figure 2. Kaplan-Meier curves of overall survival according to different groups. Survival difference of patients in group was statistically significant (p<0.001).

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Propensity score matching

After matching on sex, stage, pathology findings, and age, a total of 4,842 patients were included and divided evenly between the postoperative chemoradiotherapy (n=2421) and postoperative chemotherapy group (n = 2421). The sample included 2,779 men and 2,063 women in. There were 21 cases at T0 stage, 1,091 cases at T1 stage, 1,922 cases at T2 stage, 556 cases at T3 stage, and 1,252 cases at T4 stage. There were 2,185 cases of squamous cell carcinoma and 2,657 cases of adenocarcinoma. There were 2,300 patients aged 65 years or younger and 2,542 patients older than 65 years. The age range was 19-90 years, with a median of 66 years (mean 65.66 ± 9.72). The 5-year survival rate and median survival time before and after matching are presented in Table 2. Before matching, the median survival time was 17 months, and the 5-year survival rate was 12.55%; after matching, the median survival time was 19 months, and the 5-year survival rate was 18.5%. The prognosis of patients who had received postoperative chemoradiotherapy was significantly better than that of patients who had received postoperative chemotherapy alone (hazard ratio [HR]=0.869, 95% confidence interval [CI]: 0.817-0.925, P <0.001). Treatment regimen was an independent prognostic factor for lung cancer patients (Table 3, and Figure 3 and 4).

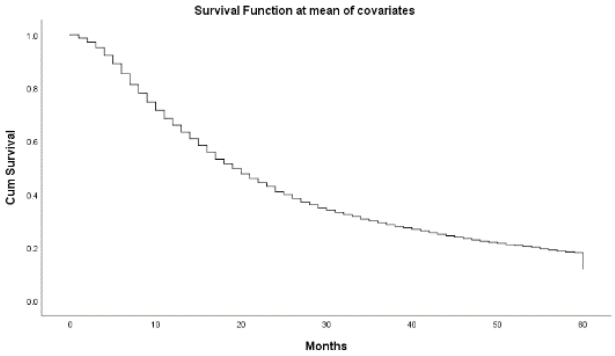

Figure 3. Survival curve at mean of covariates

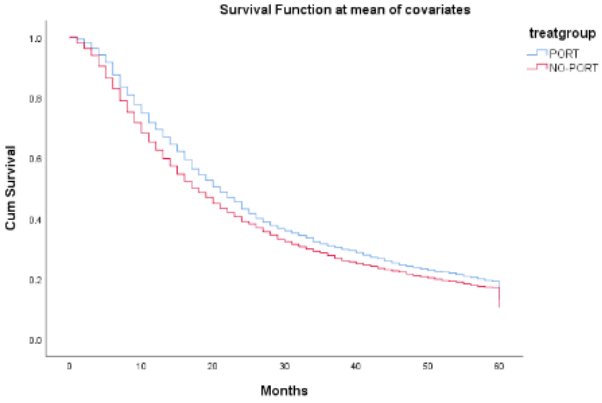

Figure 4. Significant difference survival curve of between after-matching PORT and NO-PORT (p<0.001).

Table 3. Survival outcomes for lung cancer patients included in the Surveillance, Epidemiology, and End Results (SEER) database, before and after propensity score matching

Group	Before matching				After matching			
	Cases	Percentage (%)	5-year survival rate (%)	Median survival time (months)	Cases	Percentage (%)	5-year survival rate (%)	Median survival time (months)
Sex								
Male	7119	59.33	11.14	16	2779	57.39	15.87	17
Female	4881	40.67	14.61	18	2063	42.61	22.06	22
Stage								
T0	66	0.55	30.30	37	21	0.43	38.10	28
T1	1640	13.67	19.02	23	1091	22.53	24.01	24
T2	4421	36.84	12.12	18	1922	39.69	18.21	20
T3	1842	15.35	12.87	16	556	11.48	16.19	11
T4	4031	33.59	9.95	14	1252	25.88	14.86	15
Pathology								
Squamous cell carcinoma	6652	55.43	10.34	15	2185	45.13	15.56	16
Adenocarcinoma	5348	44.57	15.30	19	2657	54.87	20.93	21
Age								
≤ 65 years	5344	44.53	14.20	17	2300	47.50	20.04	20
> 65 years	6656	55.47	11.22	16	2542	52.51	17.11	18
Treatment								
Postoperative chemotherapy + radiotherapy	2606	21.72	16.58	21	2421	50	20.12	21
Postoperative chemotherapy	9394	78.28	11.43	16	2421	50	16.89	17

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4. Discussion

NSCLC is a common type of lung cancer, of which adenocarcinoma and squamous cell carcinoma are the predominant subtypes. At the time of diagnosis, 30%-40% of patients have been reported to have disease at a locally advanced stage, accompanied by metastasis of cancer cells [4]. There are differences in the treatment of patients with N2 stage disease with mediastinal lymph node metastasis. Although comprehensive treatment is regarded as a standard regimen for N2 patients with resectable NSCLC, the optimal combination therapy regimen remains unclear [5]. Chemotherapy is required to treat resectable NSCLC [6,7]. Treatment regimens include radical concurrent chemoradiotherapy, induction chemotherapy or chemoradiotherapy, and postoperative chemotherapy or chemoradiotherapy. It remains unclear which regimen is optimal, but 5-year survival rate for any regimen is 20-45% [8]. Moreover, 5-year overall survival rates associated with micro-single-station, micro-multi-station, macro-single-station, and macro-multi-station involvement of mediastinal N2 lymph nodes have been reported as 34%, 11%, 8%, and 3%, respectively [9]. Surgery alone is insufficient, and the survival rate of patients with operable locally-advanced NSCLC is not high [10]. It is increasingly believed that patients with N2 disease stage should not be treated with surgery alone. In fact, evidence from randomized trials shows that adjuvant therapy is better than resection alone [11,12]. The guidelines on NCCN (National Comprehensive Cancer Network) treatment recommend that adjuvant chemotherapy or adjuvant chemoradiotherapy be performed for patients with disease stage N2. The present study examined differences in survival rates between patients with pN2 disease stage treated with chemotherapy alone and chemoradiotherapy.

There is little controversy around postoperative chemotherapy as a standard postoperative treatment for N2 stage NSCLC with postoperative lymph node metastasis [13]. However, controversy surrounds administration of adjuvant radiotherapy. Based on data from the National Cancer Database (NCDB), Drake et al. have reported no difference in the median survival time between patients treated with adjuvant chemoradiotherapy and patients treated with adjuvant chemotherapy after R0 resection of disease at stage N0 and pathological N2 (3.9 years vs 3.8 years, $P = 0.705$) [14]. Moreover, Spicer et al. have conducted a retrospective analysis of data from four chest tumor centers, and compared 5-year overall survival (OS) and disease-free survival (DFS) in patients treated with N2 postoperative chemotherapy and N2 postoperative concurrent chemoradiotherapy. Their study revealed no differences in recurrence rates, recurrence mode, perioperative mortality, OS, or DFS in patients who had received preoperative invasive mediastinal staging [15].

It has been reported that postoperative radiotherapy can benefit pN2 patients. The American Thoracic Society guidelines did not support administration of adjuvant radiotherapy for occult N2 (NSCLC) after RO resection. Postoperative radiotherapy increased the local control rate but did not improve the OS rate [16]. A meta-analysis of relevant studies has shown that adjuvant radiotherapy lacked survival benefits after complete resection of NSCLC compared with operation alone. In 2006, Lally et al., using the SEER data, argued that postoperative radiotherapy improved the survival rate of N2 patients but did not benefit patients with disease stage N0 or N1 [17]. Douillard et al. have retrospectively analyzed data from pN2 patients who had received postoperative adjuvant radiotherapy, revealing that postoperative adjuvant radiotherapy generated more benefits but showed a negative effect on pN1 [18]. Based on data from the National Cancer Database, Herskovic et al. have conducted a stratified analysis to examine whether 2,691 patients with negative N2 (ⅢA) resection margin who had received adjuvant chemotherapy during 2004-2013 should receive postoperative radiotherapy. In their study, the median survival time was 27.43 months and 25.86 months ($p < 0.05$), respectively. Postoperative radiotherapy significantly prolonged survival [19]. With improvements to radiotherapy technology, the local control rate increased, and treatment toxicity decreased [20]. Moreover, Su et al. have found that 1-year, 3-year, and 5-year OS rates associated with postoperative chemoradiotherapy and postoperative chemotherapy were 98.3% vs 86.1%, 71.7% vs 53.0% and 45.7% vs 39.0%, respectively ($P = 0.019$) [21].

In summary, there have been many studies aimed at examining the efficacy of N2 postoperative treatment regimens. In the present study, which involved analysis of data from the SEER database, we have shown that age, sex, disease stage, pathology type, and treatment regimen are factors that affect the prognosis of patients with N2 lung cancer. In the present study, women and patients with adenocarcinoma had a better prognosis than did men and patients with squamous cell carcinoma. These findings suggest that targeted therapy can be considered to prolong survival. Multivariate analysis has revealed that survival associated with postoperative chemoradiotherapy was longer than survival time associated with postoperative chemotherapy alone. With the development of novel immunotherapy and targeted therapy regimens, further research is needed to identify the optimal postoperative treatment regimen for N2 lung cancer.

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5. Conclusions

It can be seen from this study that the prognosis of N2 lung cancer is affected by many factors. Young, female and adenocarcinoma patients have more survival advantages. With the increase of T stage, the prognosis is worse and worse. Postoperative adjuvant radiotherapy is better than postoperative chemotherapy alone.

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Statement of Interest

There is no conflict of economic interests affecting its scientificity and credibility in this study.

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ARTICLE

Stromal and Tumor Glioma-derived Cells with Similar Characteristics Have Differences in α-Smooth Muscle Actin Expression and Localization

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ABSTRACT

Gliomas are solid brain tumors composed of tumor cells and recruited heterogenic stromal components. The study of the interactions between the perivascular niche and its surrounding cells is of great value in unraveling mechanisms of drug resistance in malignant gliomas.

In this study, we isolated the stromal diploid cell population from oligodendroglioma and a mixed population of tumor aneuploid and stromal diploid cells from astrocytoma specimens. The stromal cells expressed neural stem/progenitor and mesenchymal markers showing the same discordant phenotype that is typical for glioma cells. Moreover, some of the stromal cells expressed CD133. For the first time, we demonstrated that this type of stromal cells had the typical myofibroblastic phenotype as the α-SMA+ cells formed α-SMA fibers and exhibited the specific function to deposit extracellular matrix (ECM) proteins at least in vitro. Immunofluorescent analysis showed diffuse or focal α-SMA staining in the cytoplasm of the astrocytoma-derived, A172, T98G, and U251MG glioma cells. We could suggest that α-SMA may be one of the main molecules, bearing protective functions. Possible mechanisms and consequences of α-SMA disruptions in gliomas are discussed.

1. Introduction

Gliomas represent a heterogeneous group of brain tumors characterized by a highly aggressive nature. Malignant glioblastoma exhibits a high self-renewal and proliferation capacity of glioma stem cells and perivascular niche cells. The study of the interactions between the perivascular niche and its surrounding cells is of great value in unraveling mechanisms of drug resistance in malignant gliomas [1].

A neoplastic component of glioma consists of the transformed cells of astrocytic or oligodendrocytic origin. Like all solid tumors in addition to the neoplastic component, glioma also contains a heterogeneous stroma which is comprised of prominent vessels and recruiting cells such as antigen-presented cells, reactive astrocytes, mesenchymal stem cells (MSC), and glioma-associated stromal

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cells (GASC), termed also cancer-associated fibroblasts (CAFs) [2-4]. Neural stem or progenitor cells from the adult brain which are considered as the most putative cell of origin for glioma [5-8] may also be a part of the glioma tumor stroma. Some studies in xenograft models have shown that neural stem cells (NSC) can display extensive tropism toward the tumors [9-11]. In PDGF-induced gliomas stem or progenitor-like cells expressing neural markers can occupy large areas and even predominate in a tumor bulk [12-15]. Gene expression profiles of these cells resemble ones of tumor cells. Moreover, the ability of the stromal cells to acquire genetic alterations typical for gliomas has been shown in some experimental models [13]. Thus, identification of this type of stromal cells and obtaining its pure culture is rather difficult because it is poorly distinguishable from the tumor cells. The culture of neural stem/progenitor-like stromal cells has been obtained by using an artificial xenograft model [16]. The existence and proportion of this stromal cell component in gliomas are not clearly defined.

The role of the tumor microenvironment in cancer progression deserves significant attention. Cancer cells recruit and transform the stromal cells, which acquire myofibroblastic phenotype determined by the expression of alpha-smooth muscle actin (α-SMA) [17-22]. Thus, Öhlund et al. [19] have identified a stromal cell subpopulation with elevated expression of α-SMA located immediately adjacent to neoplastic cells in mouse and human pancreatic ductal adenocarcinoma tissue. Holm Nielsen [20] has found that the proportion of the α-SMA- positive myofibroblasts are upregulated in lung fibrosis and cancer. Vacz et al. [21] have demonstrated that the proportion of α-SMA(+)/CK(+) cells is significantly higher in colorectal cancer samples compared to healthy or adenoma samples. The myofibroblast function is associated with extracellular matrix (ECM) deposition in both normal physiological and pathological conditions [4,23]. Glia progenitor cells provide these functions in the CNS. During embryonic and postnatal development these purely differentiated cells exhibit a myofibroblastic phenotype and provide homing of neuron and glia precursors through the emerging brain areas [24-26]. In the adult nervous system reactive glia demonstrates myofibroblastic features in response to injury [27]. These cells originate from the pool of progenitor cells disseminated through the brain. Reactivated with inflammatory cues they take part in the formation of a glial scar in the central and peripheral nervous system of adults. The levels of the same inflammatory cytokines (TGFβ1, IL6, etc.) were found to be elevated in neoplastic areas of the brain [28] that can result in the appearance of myofibroblasts accumulating in the tumor microenvironment. Recruiting CAFs, MSC and reactive astrocytes could be responsible for the production and modulation of ECM in gliomas. The ECM composition and stiffness are altered in malignant gliomas and the correlation between these alterations and malignancy is discussed [29]. Whether neural stem/progenitor-like stromal cells could demonstrate myofibroblastic features and contribute to ECM deposition during gliomagenesis remains unknown.

Glioma cells according to their probable origin should have a basic opportunity to exhibit the myofibroblastic phenotype and α-SMA shift. However, it has been shown that α-SMA expression is disrupted in transformed cells [30,31]. In oncogenic transformed fibroblasts these disruptions were mediated by repression of α-SMA promoter activity with serum response elements [32]. In a transformed rat cell line α-SMA was involved in the regulation of cell growth and motility and led to the suppression of the malignant phenotype of this cell line [33]. In tumor cells from primary and secondary glioblastomas α-SMA expression is absent in the cases when the loss of heterozygosity on chromosome 10q occurs [34]. In secondary glioblastomas, the repression of the α-SMA gene may be also associated with p53 mutations as the α-SMA gene has been shown to be a transcriptional target of this tumor suppressor protein [35]. It is not yet well explored if all glioma cells are unable to express α-SMA and to acquire myofibroblastic phenotype.

In this report, we isolated the stromal cell population from oligodendroglioma as well as the stromal and tumor cells mixed population from anaplastic and fibrillar astrocytoma specimens. Both stromal and tumor glioma-derived cells showed the same molecular profile indicating that the cells belonged to the same lineage. We first demonstrated that the stromal cells expressing neural stem and progenitor markers had the typical myofibroblastic phenotype as they express α-SMA and exhibit their specific function to deposit ECM proteins at least in culture conditions. We also attempted to determine whether glioma cells retain the expression of α-SMA.

2. Materials and methods

2.1 Tumor Specimens and Glioma-derived Cell Lines

Tumor samples were obtained from three patients undergoing surgical treatment in the Polenov Neurosurgical Institute at Federal Almazov Medical Research Centre (St. Petersburg, Russia). These tumors were histologically characterized according to the WHO classification as oligodendroglioma grade II (Olig II), fibrillar astrocytoma grade II (FAII) and polymorphic anaplastic astrocytoma grade III (AAIII). After surgical removal tumor samples

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were kept in PBS at room temperature up to use and then they were washed, minced up to 1 mm^3 pieces, enzymatically treated, and passed through a syringe with a needle 20G. The obtained suspension contained single cells and undissociated tissue pieces were plated on a culture plate. These cultures were further designated as "mix" cultures. Overnight unattached tumor pieces were transferred on a culture plate. Cell lines were obtained from these cultures by outgrowth of the cells from tumor pieces. When the cells reached confluence they were trypsinized, diluted and transferred on a plate. This standard procedure was repeated for all cell lines derived from primary cultures. The cell cultures were maintained in DMEM/F12 (Lonza, Belgium) supplemented with 10% fetal calf serum (HyClone Laboratories, Utah, USA), 2.5 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin (Biolot, Russia) under standard conditions (37˚C, atmosphere of 5% CO2 in air). Permanent human glioma cell lines A172, U251MG and T98G were obtained from the Vertebrate Cell Culture Collection at the Institute of Cytology RAS (St. Petersburg, Russia) and were maintained under the same culture conditions.

2.2 Magnetic Cell Separation of CD133+ Cells

The cells were harvested and resuspended in PBS containing 0,5% BSA and 2 mM EDTA. Positive magnetic cell separation (MACS) was performed using the CD133 MicroBead Kit (Miltenyi Biotec, Germany). The ratio of CD133+ cells was estimated by cell counting.

Immunocytochemical staining cells were grown on coverslips at high or low density for the indicated periods of time, then fixed with 4% formalin (Sigma, USA), permeabilized with 0.1% Triton X-100 or 0.5% Triton X-100 for nucleolin staining, and incubated with PBS containing 1% BSA (Sigma, USA) and 2% FCS for 30 min to prevent unspecific binding of antibodies. Then, the samples were incubated with the following primary antibodies: mouse anti-α-SMA (1:300 dilution, Sigma), rabbit anti-GFAP (1:50, Sigma), mouse anti-CNPase (1:50, Chemicon), rabbit anti-nestin (1:50, Millipore), mouse anti-vimentin (1:150, Sigma), rabbit anti-collagen type I (1:40, Chemicon), mouse anti-fibronectin (1:200, Sigma), rabbit anti-CD133 (1:150, Abcam) or rabbit anti-nucleolin (1:100, Proteintech Europe) in 1% BSA overnight at 4°C. After washing with PBS samples were incubated with secondary antibodies Alexa-Fluor-488-conjugated goat anti-mouse-IgG (1:400, Invitrogen) or Atto-550 conjugated goat anti-rabbit-IgG (1:300, Sigma), followed by staining with rhodamine-phalloidin (1:40, Invitrogen) and DAPI (2 μg/ml, Sigma). Then, the coverslips were mounted in Vectashield medium (Vector Laboratories). Samples were analyzed with the confocal fluorescence microscope Leica TCS SL (Leica Microsystems, Germany).

2.3 RNA Isolation and RT-PCR

Total RNA was isolated from cells with the GenJET RNA purification kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. RNA concentration was determined with an ND1000 spectrophotometer (NanoDrop, USA). RNA solutions were stored at -80°C. Sample volumes from 0.5 to 11 μL of total RNA solution (depending on RNA concentration) were used in a volume of 20 μL for each reaction of the reverse transcription. The reaction was performed with a RevertAid reverse transcriptase kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions using random hexamer primers. cDNA was stored at -20°C. PCR was performed at a volume of 25 μL with DreamTaq DNA polymerase (Thermo Scientific, Lithuania) according to the manufacturer's instructions. DNA was amplified for 35 cycles. PCR products were analyzed by electrophoresis in 2% agarose gel. Gel image acquisitions were made with the ChemiDoc system (Bio-Rad, USA). Primers were designed using primer-BLAST resources of the National Center for Biotechnology Information (NCBI) and an IDT OligoAnalyzer (http://eu.idtdna.com/calc/analyzer). Primers for vimentin gene identification corresponded to the reported sequences (Velpula et al., 2011). The following primers sequences were used:

Vimentin, 5′-GAACGCCAGATGCGTGAAATG-3′ (forward),
5′ CCAGAGGGAGTGAATCCAGATTA-3′ (reverse);
CD44, 5′ AAGGTGGAGCAAACACAACC-3′ (forward),
5′-ACTGCAATGCAAACTGCAAG-3′ (reverse);
GFAP 5′-GCACGCAGTATGAGGCAATG-3′ (forward),
5′-TAGTCGTTGGCTTCGTGCTT-3′ (reverse);
NG2, 5′-GGTGGTTTCAGATCGGGAGG-3′ (forward),
5′ CAGTGACGTTCACTACGGCT-3′ (reverse);
CD133, 5′-GGTGCTGTTCATGTTCTCCA-3′ (forward),
5′ ACCGACTGAGACCCAACATC-3′ (reverse);
β-actin, 5′-TTCCTGGGCATGGAGTCCT-3′ (forward),
5′-AGGAGGAGCAATGATCTTGATC-3′ (reverse).

2.4 Fluorescence-activated Cell Sorting Analysis

The cells were grown to 80-90% confluence, trypsinized with 0.25% Trypsin-EDTA (Gibco), washed with PBS, centrifuged at 1,000 rpm for 5 min, and the pellet was resuspended at a concentration of 1 x 10^6/mL. Glioma cells were stained with fluorochrome-conjugated antibodies for at least 30 minutes at 4 °C and then the samples were diluted by FACS buffer (PBS with 1% BSA and 0.05% sodium azide). The analyses were made on a flow

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cytometer CyFlowSpace (Partec, Germany) and ≥10,000 events were collected in each analysis with forward scatter and side scatter. Data were analyzed by FloMax 2.82 software (Partec, Germany). The following antibodies were employed: CD44-FITC, CD73-PE, CD105-PE, CD90-FITC, CD29-PE, CD13-PE, CD9-FITC, CD130-FITC, CD146-PE, CD10-FITC, CD11b-PE, CD45-FITC, and HLA-DR-PE (Beckman Coulter, BD Pharmigen, Chemicon, Caltag Laboratories, Becton Dickinson, and eBioscience). Mouse IgG-FITC and IgG-PE isotype controls (DAKO) were used for assessing the background staining of cells.

For DNA content analysis, cells were suspended in 300 µl PBS containing 200 µg/ml of saponin (Fluka, NY, USA), 250 µg/ml RNase A (Sigma, St. Louis, MO, USA, number R4642), and 50 µg/ml PI, incubated for 60 min at room temperature and subjected to FACS analysis. At least 10,000 cells were measured per sample. Normal human lymphocytes were used as internal diploid control. Samples were analyzed by flow cytometer CytoFLEX. Histograms were prepared using CytExpert program version 1.2 (Beckman Coulter, Brea, CA, USA). DNA index (DI) was determined as the ratio of the mean DNA content of the glioma-derived cells to the mean DNA content of the control diploid cells.

2.5 Western Blotting

Total protein was isolated from monolayer cells at 72 h after plating. Briefly, the cells were washed with PBS and collected in RIPA lysis buffer containing a protease inhibitor cocktail (Sigma). The lysates were clarified by centrifugation at 14,000×g at 4°C for 15 min. Protein concentrations were determined by the Pierce BCA protein assay kit (Thermo Fisher Scientific) with BSA as a standard, and an equal mass of proteins from the cell samples were resolved on a 10% SDS polyacrylamide gel. In the case of analysis of collagen type I and α-SMA in glioma cell lines 25 µg of protein per lane was applied, in all other cases - 10 µg of protein per lane. After that, the proteins were electrotransferred onto a nitrocellulose membrane (Biorad). After blocking in PBS buffer with 0.05% Tween-20 (PBS-T) containing 5% nonfat dry milk at room temperature for 1 hr, blots were incubated overnight at 4°C with monoclonal anti-α-SMA antibodies (1:1000, Sigma, A5228), or polyclonal anti-β-tubulin antibodies (1:1000, Abcam, AB6046) or polyclonal anti-collagen type I antibodies (1:200, Chemicon, AB745) in blocking buffer. After three washes with PBS-T, the membranes were incubated for 1 hr at room temperature with rabbit peroxidase-labeled anti-mouse-IgG secondary antibodies (1:20000, Thermo Fisher Scientific, #31431) or with goat peroxidase-labeled anti-rabbit-IgG secondary antibodies (1:20000, Sigma, A0545) in blocking buffer. Membranes were washed three times with PBS-T and then were incubated in the chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific, #34095) and visualized on Fusion FX imager (Vilber Lourmat). Densitometric analysis of the bands was performed by employing Fusion-FX software (Vilber Lourmat).

3. Results

The stromal cells growing from tumor pieces together with tumor cells are not MSC, microglia/macrophages, or endothelial/subendothelial cells

The cells were isolated from glioma specimens by different approaches and compared cellular morphology and phenotype. The glioma-derived cultures obtained from the fraction of tumor pieces by cell sprouting presented morphologically homogeneous cell populations. At early passages, FAII- and AAIII -derived cells had polygonal epithelial-like shapes whereas ones derived from OligII had elongated fibroblast-like morphology (Figure 1). Further, the cells were enlarged and acquired a flattened shape.

To characterize the cell cultures and estimate their homogeneity we used flow cytometry for cell surface markers which are generally expressed on glioma cells and cells relating to the tumor stroma such as microglia, macrophages, pericytes, endothelial, smooth muscle, and

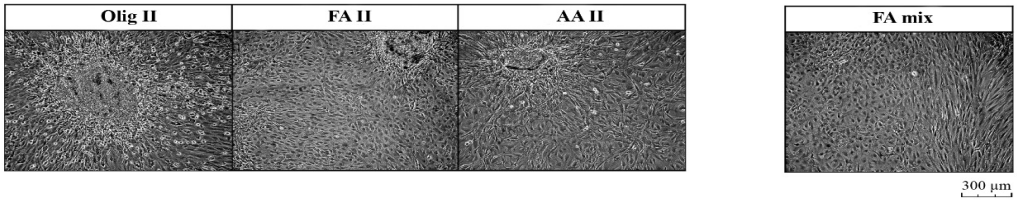

Figure 1. Phase-contrast images of oligodendroglioma grade II (OligII), fibrillar astrocytoma grade II (FAII), anaplastic astrocytoma grade III (AAIII)-derived cells grown out from tumor micro fragments, and cells representing a mixed fraction of single cells and tumor pieces (FAII mix).

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mesenchymal stem cells (Table 1).

Table 1. Phenotypic analysis of FAII, FAII mix, AAIII, AAIII mix, and A172 cells by flow cytometry.

	FAII	FAII mix	AAIII	AAIII mix	A172
CD44	99,6	99,8	97,5	94	98,4
CD73	99,9	99,7	99	75	4,8
CD105	99,7	99,7	95,5	71	39,8
CD90	4,4	61	3,6	1,2	21
CD29	99,8	99,9	93	93	98
CD13	99,8	94,6	99,5	96	13
CD9	100	99,8		71	87
CD146	5,8	23	2,9	2,8	97
CD130	1,1	0,4	1,6	1	7,3
CD10	0,2	0,1	3,2	0,7	
CD11b	0,2	1,6		20	
CD45	0,2	0,2			
HLA-DR			3,5	25,5	

Cells were immunostained with antibodies against mesenchymal markers (CD44, CD73, CD105, CD90, CD29, and CD13), endothelial, subendothelial and mesenchymal marker CD146, microglia/macrophages markers (CD11b, CD45 and HLA-DR), CD9, CD130, and CD10.

FAII- and AAIII -derived cells showed a similar surface molecular pattern: high level of mesenchymal markers CD44, CD73, CD105, CD29, CD13, but low CD90; low level of CD146, the laminin receptor, which is typical for pericytes, endothelial, smooth muscle and also mesenchymal stem cells; negligible levels of microglia/macrophages markers CD11b, CD45. For comparison, we analyzed the A172 permanent cell line and revealed a modified pattern of mesenchymal markers, e.g. low percentage of CD73+ and CD13+ cells that may be the result of the long-term culturing and clonal cell selection in permanent cell lines. This assumption is consistent with the detection of high expression of CD146 marker in A172 permanent cell line since this molecule is associated with clonal properties [36].

Flow cytometry analysis of FAII mix and AAIII mix cultures obtained from the mixed fraction of single cells and tumor pieces revealed double peaks on graphs for a number of surface markers whereas only one peak for the corresponding markers was revealed in FAII and AAIII cultures obtained from tumor pieces only (Figure 2A, B). Thus, FAII mix culture contained 61% CD90+ cells, most likely MSCs, though almost no CD90 expressing cells were present in FAII culture (Figure 2A). Apparently, these stromal cells could be detected visually as elongated cells grouped together in FAII mix culture (Figure 1). HLA-DR positive cells presumably representing microglia/macrophages dominated in AAIII mix culture compared to AAIII culture (25% vs 3.5%) (Figure 2B). Therefore, cells grown intensively from the tumor pieces arise to a morphologically homogeneous cell population depleted for MSC and microglia/macrophages that appear to be a significant part of the cell culture obtained from completely dissociated tumor tissue.

In spite of morphological and surface marker homogeneity of FAII and AAIII cell cultures, the analysis of DNA content by flow cytometry revealed two distinct cell populations: normal stromal diploid and tumor aneuploid (Figure 2C). Aneuploid cell populations in both astrocytoma-derived cell lines were nearly tetraploid (DI was 1,86 (P2) and 1,85 (P6) for FAII, and 1,88 (P5) and 1,89 (P15) for AAIII), the second histogram peak in the PI channel coincides with overlapping aneuploid (G1/G0) tumor cell population and the diploid 4N (G2/M) stromal cell fraction. Interestingly, the proportion of aneuploid and diploid cells remained nearly constant over the passages and was higher in AAIII than FAII cell culture. It may reflect the mutual influence of the tumor and stromal cells and the establishment of a stabilized ratio of the cell types in culture. According to the DNA assay, OligII cells were all diploid (Figure 2C) pointing to the absence of neoplastic component. It is consistent with reports where it has been demonstrated that oligodendroglioma tumor cells are not viable in culture under adherent conditions [16,37].

The stromal cells are α-SMA+ and responsible for the deposition of collagen type I and fibronectin. Alpha-smooth muscle actin (α-SMA) is regarded to be one of the most significant markers of stromal cells in gliomas [2]. Immunostaining with monoclonal antibody to α-SMA confirmed the presence of a stromal component in astrocytoma-derived cell cultures FAII and AAIII (Figure 3A). Numerous cells with well-organized α-SMA-filaments were seen (α-SMA+ cells). Of note, cells without α-SMA-filaments had a diffuse cytoplasmic α-SMA staining. OligII-derived cell culture was a total α-SMA+ stromal cell population according to immunofluorescence (Figure 3A). Western blot analysis revealed the highest level of α-SMA in OligII cells (Figure 3B). Interestingly, FAII mix culture in which MSCs were about 60% of the total cell population showed α-SMA level lower than FAII cells (Figure 3B) in which MSC were only about 4% of the total cell population according to flow cytometry (Figure 1C). This fact suggests that there are neural stem/progenitor-like stromal cells in glioma that produce more α-SMA than MSCs.

In order to estimate the specific fibrotic potential of α-SMA+ cells, the intracellular synthesis of collagen type I and fibronectin and their deposition in ECM were compared between glioma cell cultures by immunofluorescence (Figure 3D).

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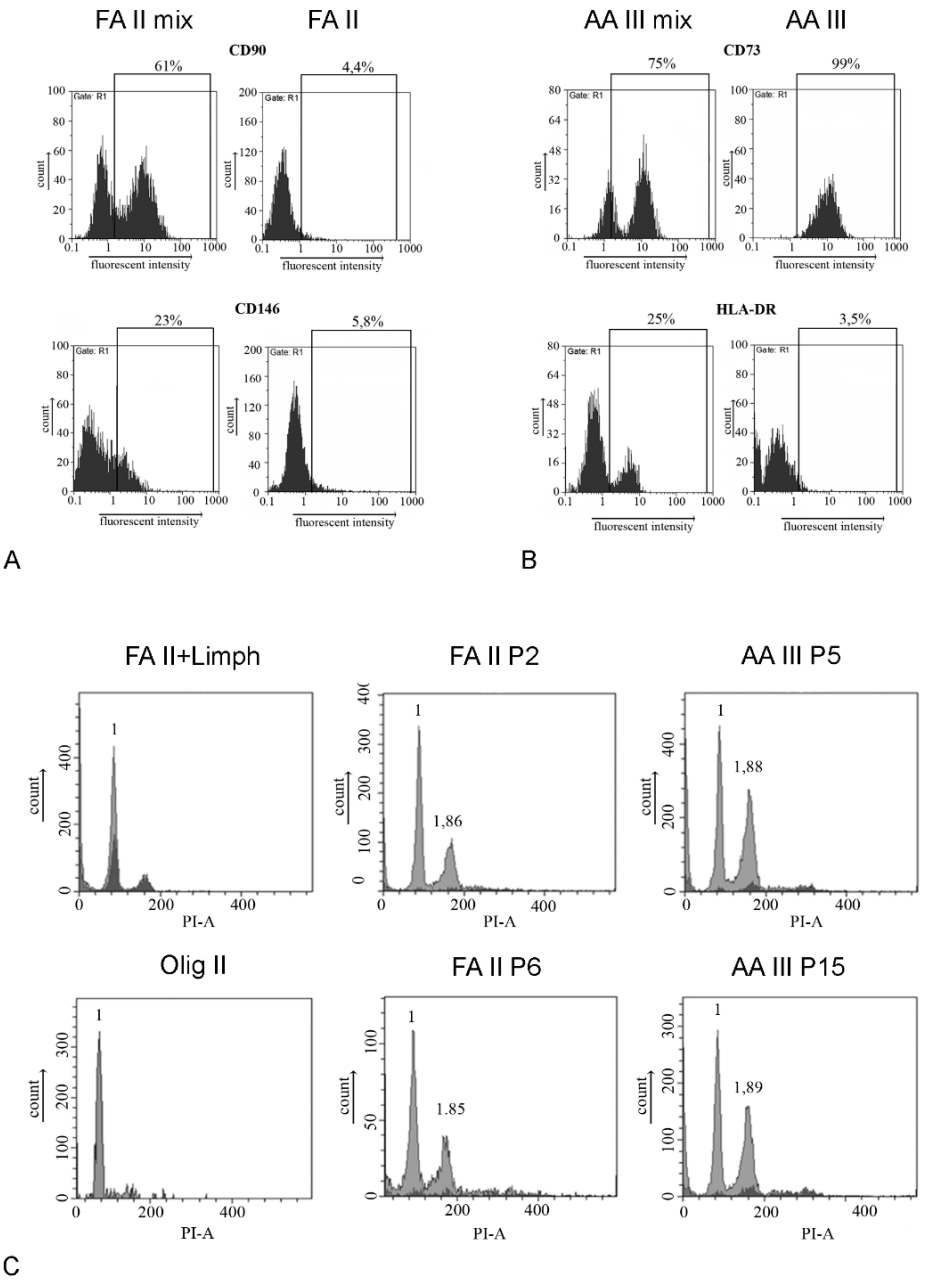

Figure 2. Phenotypic characterization of cells derived from glioma specimens.(A) Flow cytometry assay of CD90 and CD146 expression in FAII mix and in FAII cultures. (B) Flow cytometry assay of CD73 and HLA-DR expression in AAIII mix and AAIII cultures. (C) DNA assays of cells derived from glioma specimens. Flow cytometry histograms showing the DNA content in FAII, AAIII, and OligII cells. Stromal cells and lymphocytes were DNA diploid (DI=1). Tumor cells were aneuploid: FAII (DI=1,86 (2P) and 1,85 (6P)) and AAIII (DI=1,88 (5P) and 1,89 (15P)).

According to immunofluorescence at the 15-th day of culturing the deposition of collagen type I and fibronectin was more abundant in OligII culture (Figure 3D). FAII and AAIII cultures showed resembling the pattern of protein deposition with an increased level in FAII culture. These results suggest that there is a positive correlation between the amount of α-SMA+ cells and collagen type I and fibronectin deposition. These results also show that the ability of glioma-derived stromal cells to produce ECM may have a tendency to decline with malignancy development.

Glioma cells usually do not express α-SMA and show reduced deposition of collagen type I and fibronectin

As NSC from the subventricular zone and glia progenitors from brain parenchyma are believed to be the cell

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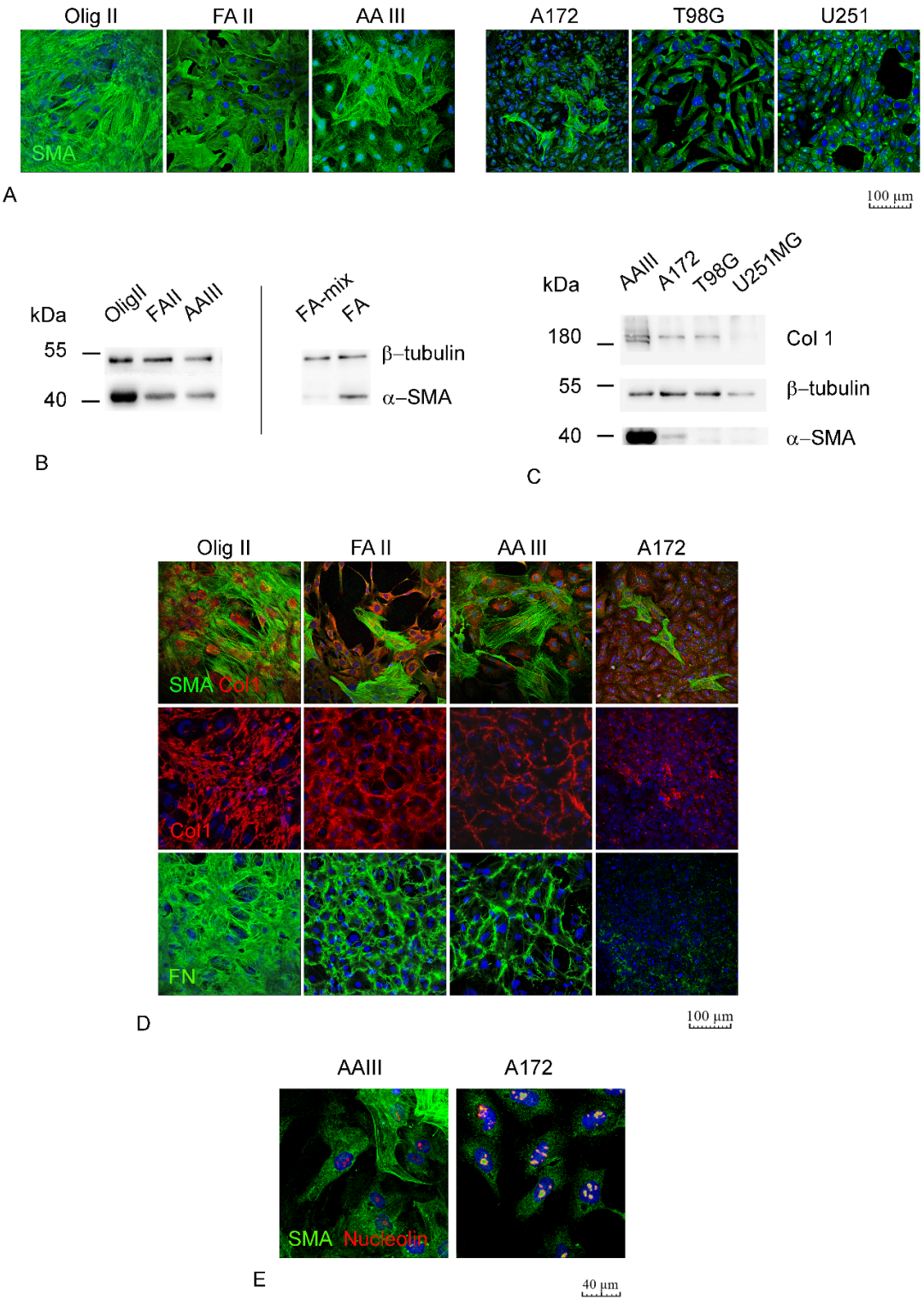

Figure 3. α-SMA+ stromal cell component of primary cultures and permanent glioma cell lines, their fibrotic potential. (A) Immunocytochemical staining of α-SMA in OligII, FAII, and AAIII cultures and glioma cell lines. (B) Western blot analysis of α-SMA in OligII, FAII, AAIII, FAII mix cells and glioma cell lines. (C) Western blot analysis of α-SMA and collagen type I in AAIII culture and glioma cell lines. (D) Immunocytochemical staining of α-SMA and collagen type I (upper horizontal line) intracellularly after 3 days in culture, and collagen type I (central horizontal line) and fibronectin (bottom horizontal line) extracellularly after 15 days in culture. (E) Double immunocytochemical staining of α-SMA and nucleolin in AAIII and A172 cells. Localization of α-SMA in nucleoli of A172 and no staining in the AAIII cells.

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of origin for glioma [5-8] and can express α-SMA [24-26], we attempted to determine whether glioma cells express α-SMA. Immunostaining of α-SMA in three permanent glioma lines A172, T98G, and U251MG revealed that the A172 cell line contained a few α-SMA+ cells with their numbers increasing over the time spent in culture (Figure 3A). A172 cells without α-SMA fibers showed negligible α-SMA staining in the cytoplasm and distinct spotted signal in the nucleus. The presence of α-SMA+ cells pointed to the basic opportunity of glioma cells to synthesize α-SMA and became additional proof for the neural stem/progenitor cell nature of glioma cells. Although T98G and U251MG lines did not have cells containing α-SMA-filaments they exhibited a homogeneous strong α-SMA staining in the cytoplasm as well as a spotted α-SMA staining in the nucleus (Figure 3A).

Western blot analysis revealed a very weak α-SMA band in A172 cells that apparently correspond to α-SMA+ cells, and did not reveal α-SMA full-length protein (43 kDa) in T98G and U251MG lines in which the α-SMA+ cells were absent (Figure 3C). Taking into account that T98G and U251MG cells have mutations and A172 cells have a deletion in tumor suppressor PTEN gene [38] which is located side by side with Acta2 gene encoding α-SMA, we speculate that α-SMA protein could also be altered so that F-actin assembly was disrupted in permanent glioma lines. We speculate further that this altered protein of α-SMA might not be revealed in denatured form by electrophoresis but might be stained in native form by immunocytochemistry.

In order to estimate the fibrotic potential of tumor cells, the intracellular synthesis of collagen type I and fibronectin and their deposition in ECM were compared between AAIII cells and A172, T98G, and U251MG cell lines by Western blotting and immunofluorescence (Figure 3C, 3D). Western blot analysis revealed a more reduced intracellular synthesis of collagen type I in the permanent cell lines compared to AAIII cells (Figure 3C). The deposition of collagen type I and fibronectin by A172 cells at the 15-th day of culturing was also significantly reduced compared with glioma-derived cells (Figure 3D).

Because a strong α-SMA spotted positivity was observed in the nucleolar area in all permanent cell lines, we performed co-immunostaining of α-SMA with nucleolin as a marker for nucleoli (Figure 3E). The results clearly showed that nuclear α-SMA was localized exclusively in the nucleoli. Of note, the α-SMA+ A172 cells also exhibited distinct nucleolar α-SMA localization. Nucleolar localization of α-SMA was also detectable in glioma-derived cells, although the fluorescent intensity was much lower, sometimes barely visible. These results suggest that α-SMA-altered forms could perform cellular functions differently from wild-type α-SMA. Nucleolar localization of α-SMA could be associated with clonal properties of the long-term culturing permanent cell lines and, therefore, with a more proliferative phenotype. Similarly, nucleolar localization of many proteins occurs in highly proliferating cells. Furthermore, the presence of β-actin and non-muscle myosin 1 was shown in the nucleoli of HeLa cells where they were involved in the regulation of RNA synthesis [39].

The stromal glioma-derived cells express neural stem/progenitor, glial and mesenchymal markers

As α-SMA+ stromal cells growing from pieces of tumor tissues together with glioma cells did not have a mesenchymal stem or endothelial/subendothelial cell nature according to FACS analysis (Figure 1B), we assumed their neural stem/progenitor cell origin. Immunocytochemical staining of GFAP and CNPase, astrocyte and oligodendrocyte markers respectively, revealed co-expression of these glia proteins in all cells of glioma-derived cell cultures pointing to their glia precursor cell origin (Figure 4A). CNPase, the enzyme related to multiple cellular functions, displayed a distinct fluorescent signal and was typical for glial cells mitochondrial localization. Well-organized GFAP intermediate filaments were mostly seen in OligII cells. The majority of the astrocytoma cells showed diffuse staining of GFAP. RT-PCR amplification revealed expression of glia progenitor's markers GFAP, NG2, as well as mesenchymal markers vimentin and CD44 in the glioma-derived cell cultures (Figure 4B).

To be sure that α-SMA+ stromal cells indeed produce the neural stem/progenitor cell proteins, double immunostaining of α-SMA together with proteins related with low or either high malignant phenotype was performed. GFAP, the astrocyte marker, was stained in all α-SMA+ cells of all cultures, and even a strong staining signal was revealed in few cells (data not shown). Co-staining with nestin, the protein associated with high malignancy, revealed ambiguous results (Figure 4C). On the one hand, OligII α-SMA+ cells co-expressed nestin highly. The AAIII and FAII astrocytoma cells showed an unaltered or decreased level of nestin staining in α-SMA+ cells. Cells without α-SMA synthesis found only in AAIII culture showed a high level of nestin. This tumor cell population may potentially have more invasive features.

Furthermore, RT-PCR analysis revealed that all glioma-derived cell cultures expressed transcripts encoding CD133 which are specific for cancer and neural stem cells (Figure 4B). We performed magnetic cell sorting of AAIII and OligII cultures and revealed that AAIII-CD133- and

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OligII-CD133-enriched fraction reached no less than 10% of the total cell population. Following immunofluorescence showed that the cells displayed typical membranous fluorescent signal as well as distinct atypical nuclear localization of CD133. Unexpectedly, the AAIII-CD133-enriched fraction turned out similar to AAIII culture in terms of the presence of α-SMA+ cells. Double immunostaining of α-SMA and CD133 showed co-expression of these proteins (Figure 4D). However, α-SMA+ cells showed often more weak or negligible dotted signal of CD133 than cells with diffuse staining of α-SMA. Thus, CD133 expression seems to be inhibited in the α-SMA+ astrocytoma-derived cells. However, CD133+ OligII stromal cells that are total α-SMA+ cell population according to previous results (Figure 3A) showed distinct dotted signal in nuclei. Despite these differences, taken together, these results demonstrate that the stromal cells showed phenotypic mimicry with tumor cells.

4. Discussion and Conclusions

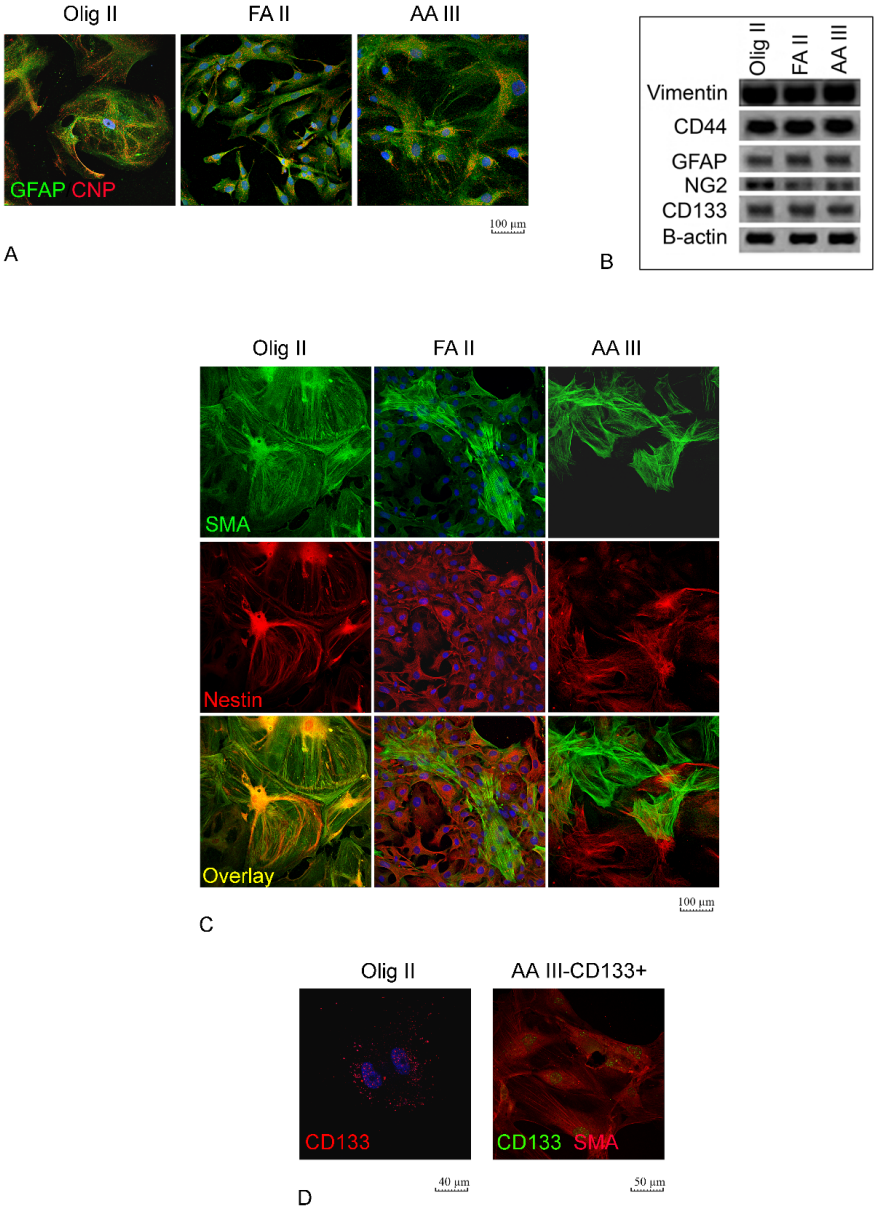

Figure 4. Expression of neural stem/progenitor, glial and mesenchymal markers in glioma-derived cells. (A) Co-expression of GFAP and CNPase in OligII, FAII, and AAIII cells. (B) RT-PCR assay of expression of mesenchymal, glial progenitor's markers and CD133 in the glioma-derived cultures. (C) Co-expression of α-SMA and nestin in glioma-derived cells. α-SMA expression is absent in some the nestin-positive AAIII cells. (D) Expression of CD133 in OligII stromal cells and in α-SMA+ cells from CD133-enriched AAIII culture.

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In this study, we isolated a mixed population of tumor and stromal cells which rapidly grew out from the tumor micro fragments. They shared a similar phenotype and were undistinguishable morphologically at the zero passage. As the tumor cells, the stromal cells showed a high expression of mesenchymal markers vimentin and CD44 as well as nestin, the protein associated with neuroepithelial development. Additional expression of both GFAP and CNPase, astrocytic and oligodendrocytic markers respectively, and other glia progenitor protein such as NG2, demonstrated the same discordant phenotype for the stromal cells as glioma cells have [40]. Moreover, the stromal cells expressed CD133, the protein associated with stemness which is not found in MSC and GASC [41-43]. All these facts indicate that this type of stromal cells could originate from the same lineage as the tumor cells.

NSCs from the subventricular zone and glia progenitors from subcortical white matter are discussed as the most likely cell of origin for glioma [5-8]. After an expansion of transformed cells, "normal" neural stem or progenitor cells may be induced to proliferation by glioma cells and migrate together with them to form tumor mass. The presence of NSCs in the tumor bulk has been revealed in PDGF-induced gliomas, along with that tropism of "normal" NSCs and glia progenitors from the adult brain to glioma was demonstrated in orthotopic xenograft models [12-15]. The stromal cells may constitute a considerable part of the tumor mass. Thus, it has been shown that 90% of glioma specimens are composed of the cells that expressed A2B5, the marker of glia progenitors from the adult forebrain [6,7]. At the same time, mimicry of many glioma-associated markers can make them poorly distinguishable by immunohistochemical analysis. We first showed that the stromal cells with neural stem/progenitor-like cell phenotype can be easily isolated from tumor specimens together with tumor cells and expanded in culture. Interestingly, the ratio of tumor and stromal cells maintained stable through many passages with the predominance of stromal cells. This could result from the significant growth potential of both cell types maintained by crosstalk between them.

Myofibroblastic phenotype accompanied by α-SMA expression is the main sign of the stromal cells in glioma-derived cultures. This feature of the stromal cells with neural stem/progenitor-like cell phenotype is not contradictory considering that there are glia progenitor cells in the embryonic and neonatal brain that express α-SMA and exhibit myofibroblastic features [24-26]. Other cells of neural origin capable to produce α-SMA are reactive astrocytes from the adult brain that can gain myofibroblastic phenotype induced by inflammatory cues in response to injury [27]. A high concentration of the same inflammatory cytokines is detected in glioma bulk and peritumoral space and may stimulate the formation of cells with myofibroblastic phenotype. However, α-SMA immunoreactivity is almost exclusively revealed in the blood vessels on immunohistochemical sections of glioma [16]. It means that our finding is a cultural phenomenon and *in vivo,* the stromal cells with neural stem/progenitor-like cell phenotype either do not switch on the α-SMA expression or express this protein on a very low level. But we assume that even with the low level of α-SMA expression the stromal cells in glioma may maintain deposition of ECM and contribute to fibrotic processes in glioma.

The expression of α-SMA is thought to be absent in glioma cells, but taking into consideration a possible origin of glioma cells from NSC or glia progenitors we studied whether glioma cells could express this protein. We found that tumor cells showed diffuse α-SMA immunostaining in the cytoplasm from very weak in A172 cells and moderate in astrocytoma-derived tumor cells up to strong in T98G cells. Moreover, a few of the A172 cells demonstrated the ability to form α-SMA filaments. U251MG cells had strong focal α-SMA staining. In addition, distinct spotted staining in the nucleoli was seen in permanent cell lines. The resembling variable diffuse or focal cytoplasmic staining of α-SMA has been revealed in neoplastic cells of gastrointestinal stromal tumors [43,44]. HeLa cells show a characteristic spot-like pattern of β-actin and non-muscle myosin 1 in the nucleoli where the actin-myosin complex has been shown to regulate mRNA synthesis [26]. Nevertheless, western blot analysis did not reveal full-length α-SMA (43kD) in T98g and U251MG cell lines in which the α-SMA+ cells are absent. In T98g and U251MG cells, α-SMA could be represented by altered monomeric forms which are not recognized by antibodies against wild-type α-SMA during western blot analysis. This assumption is consistent with the fact that the ACTA2 gene encoding α-SMA is located on a long arm of chromosome 10 in one of the most mutagen regions typical for gliomas [34,45,46]. Anaplastic astrocytomas and glioblastomas progressing from low-grade astrocytomas often show a reduced number of chromosome segments on 10q [47]. It is also noteworthy that the gene of tumor suppressor, PTEN, which is the most frequently lost in gliomas [46,48], is adjacent to the ACTA2 gene [45]. Like PTEN, the ACTA2 gene must have massive mutagenic pressure. In particular, all three permanent glioma cell lines used in the current work have abnormal PTEN alleles [38]. Mutations or deletions of α-SMA gene in gliomas could disrupt the filament formation in the same way as it occurs in smooth muscle cells or as it has been demonstrated for β-actin [50-52].

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It is remarkable that the p53 tumor suppressor has the ability to directly activate the transcription of the α-SMA gene [35]. Thus, the mutations in the p53 gene can affect α-SMA expression and they are actually the genetic hallmarks of secondary glioblastomas. At the same time, the entire loss of chromosome 10 containing the ACTA2 gene is typical for primary glioblastomas [34,53-54]. Taken together these facts may imply that the loss or decrease of α-SMA expression with disruption of fiber assembly are factually obligate for glioblastomas and seems to play a critical role in malignant transformation. Indeed, α-SMA inhibits both migration and proliferation of normal and transformed cells due to the forming of focal adhesions and by preventing the activation of small GTPase Rac1 [55-56]. At the same time, the deposition of ECM which is an attribute of α-SMA+ cells in many tissues can also inhibit migration [57-58]. Loss of α-SMA studied in ACTA2 mutant smooth muscle cells induced their proliferation through FAK and Rac1 activation, translocation of p53 from the nucleus to the cytoplasm, and increased expression and ligand-independent activation of PDGF receptor β [59]. On the other hand, in the case of disruption of microfilament bundle assembly due to missense ACTA2 mutations, an increased pool of monomeric actin leads to additional proliferative response through binding G-actin with MRTF-A, a member of the myocardin family of transcriptional coactivators. Thereby it allows SRF to bind to growth responsive genes [52,60]. The proliferative pathways induced by monomeric α-SMA might be the same in malignant cancer cells. In addition, monomeric α-SMA apparently stimulates motility: the presence of α-SMA has been shown in the leading edge of astroglia lamellipodia [25]. Therefore, we hypothesize that the existence of monomeric α-SMA in the tumor cells of anaplastic astrocytomas and benign gliomas could be a significant pathogenetic and prognostic sign.

There is a possibility that similar disruptions of α-SMA expression occur in the stromal component of gliomas. Fomchenko et al. [13] have demonstrated that genetic aberrations typical for glioma cells may be acquired by normal cells recruited into the tumor. Disruptions of α-SMA expression imply that myofibroblastic functions including ECM deposition can be impaired. ECM in turn may restrain tumor spread and so the impairment of its deposition facilitates tumor growth. Growing disturbances of α-SMA expression in stromal cells with increasing malignancy grade would induce their proliferation and, in fact, remove the main differences between stromal cells with neural stem /progenitor-like phenotype and glioma cells.

In this report, we have raised the question about a possible role of α-SMA breakdown in gliomagenesis. Taken together our findings and published literature data suggest that α-SMA may be one of the main molecule bearing protective functions, and its loss, as well as microfilament assembly disruptions in tumor and stromal cells, seems to be fatal in the way of glioma progression.

Ethics Approval and Consent to Participate

This study was conducted with the approval of the ethics committee of the Polenov Neurosurgical Institute at Almazov National Medical Research Centre and all participants signed an informed consent document.

Consent to Publish

All participants signed an informed consent document.

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Authors' Contributions

IC, IG, VP, ET carried out the study concept, design and analysis and interpretation of all data; VZ did the flow cytometry and analysis of data. IC and AM did the microscopy and histological analysis of data; YL were involved in the treatment of patients and interpretation of clinical data. IG, IC, ET, SK, SP, and AP drafted the manuscript. All authors participated in final approval of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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ARTICLE

Fertility Cancer and Hereditary Risks in Soil Sample of Nasarawa, Nasarawa State, Nigeria

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ABSTRACT

A survey of Fertility Cancer and Hereditary Risks in Soil Sample of Nasarawa was carried out. This study assessed the level of Fertility Cancer and Hereditary Risks from the naturally occurring radionuclides; ^{232}Th, ^{226}Ra and ^{40}K. 12 soil samples collected from the respective part of the Nasarawa were analyzed using the gamma-ray spectrometry NaI (Tl) detector system. The mean concentration for ^{40}K was 645.29 ± 07.32 Bq/kg, for ^{226}Ra was 28.43 ± 4.8422 Bq/Kg and for ^{232}Th was 66.84 ± 2.0201 Bq/Kg. The average effective dose due to the ingestion was 0.36±0.1 μSv/y which was approximately 1000 times lower than the world average effective dose. Radium equivalent activity Ra_{eq} (Bq/kg), alpha index and total cancer risk were found to be 161.44±8.08 Bq/kg, 0.142±0.02 and (0.21±0.05) $\times 10^{-5}$ respectively. UNSCEAR/ USEPA stipulated that; radium equivalent activity, alpha index, effective dose and total cancer risk should not exceed the limit of 370 Bq/kg, unity, 300 μSv/y and 1 $\times 10^{-4}$ respectively. Hence the values obtained in this work were within the acceptable limits. This implies that the ingestion or inhalation of soil is not associated with any radiological risk of concern.

1. Introduction

The natural terrestrial γ-radiation dose rate is important to the average dose rate received by the world's population [1,2].

Estimation of radiation dose distribution is important in assessing the health risk to a population and serve as the reference in documenting changes to environmental radioactivity in soil due to anthropogenic activities [2].

Human beings are exposed outdoors to the natural terrestrial radiation that originates predominantly from the upper 50cm of the soil [3].

Only radioactivity with half-lives comparable with the age of the earth or their corresponding decay products

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existing in terrestrial material such as ^{232}Th, ^{226}Ra and ^{40}K are of great interest. Since these radionuclides are not uniformly distributed, the knowledge of their distribution in soil and sediments plays an important role in radiation protection and measurement [4].

Gamma radiation from these represents the main external source of irradiation to the human body and the concentrations of these radionuclides in soil are determined by the radioactivity of the rock and nature of the process of the formation of the soils [5,6].

Therefore, radionuclides in soil generate a significant component of the background radiation exposure to the population [7].

The aim of this work is to measure the specific activity of the naturally occurring radionuclides (^{40}K, ^{226}Ra and ^{232}Th) in different types of soils from Nasarawa in Nasarawa State using Sodium Iodide-Thalium Gamma Spectroscopy System.

The objective of this work will be accomplished through the following types of measurement: Radionuclide Activity Concentrations in surface soil, Radium Equivalent Activity, Annual Effective Doses, Alpha Index (I_α) and Cancer and hereditary risks of the studied area.

The area of toxic and water pollutants has been the subject of interest and concern for many years. The assessment of impact on human health aids major decisions on control of population by Federal, State and Local Governments. This will be an outcome of this study. This study shall identify the areas, and the level of radiation present in the areas, which is Nasarawa, Nasarawa State, Nigeria. Radioactive material can remain dangerous for long periods, which requires radioprotection measures in order to protect the health of the workers and the public in general.

The primary parameter that determines the environmental health effects of radioactive particles and their concentration, decay rate and chemical composition. These parameters, however, are spatially and temporally variable. The identification and quantification of natural radioactivity represent demanding analytical challenges. This study shall outline the study perspectives on the properties and interactions of natural radioactivity and their effects on environmental and human health. At the end of the study, there would be a multi – disciplinary benefits and applications. The study shall serve as an academic reference material and can contribute significantly to knowledge especially as regard to health and environment.

This work focused only on some selected mining areas of Nasarawa in Nasarawa State, Nigeria. The work will give detailed information on natural radioactivity concentration in the study area, as well as discussing the protective measures that must be taken to regulate or prevent people from high dose of radiation.

2. Methodology

2.1 Soil Samples Collection

Four sample locations were chosen from all over Nasarawa in Nasarawa State, Nigeria, to conduct the radiometry study. Three samples were collected from each sample area to make twelve samples of soil. The samples were collected at 0.5 m depth level from the surface of the soil. From each area, as stated earlier, three samples were collected. Firstly, from the mining spot, secondly from a distance of 100 m away from the mining spot, and thirdly, from the river area within the mining spot. The collected samples were then sealed in a labeled polythene bags and enclose into one sack for easiest transportation from the mining or sample point to the house.

2.2 Soil Sample Preparation

The collected samples (soil) brought into the laboratory are left open (since it is wet) for a minimum of 24 hours to dry under ambient temperature. They were grounded using mortar and pestle and allowed to pass through 5 mm-mesh sieve to remove larger object and make it fine powder. The samples were packed to fill a cylindrical plastic container of height 7 cm by 6 cm diameter. This satisfied the selected optimal sample container height. Each container accommodated approximately 300 g of sample. They were carefully sealed (using Vaseline, candle wax and masking tape) to prevent radon escape and then stored for a minimum of 24 days. This is to allow radium attain equilibrium with the daughters.

2.3 Soil Sample Analysis

Gamma-ray spectrometry technique was employed in the spectral collection of the prepared sample using the higher energy region of the gamma-lines. This consists of a 7.62 cm by 7.62 cm NaI (Tl) detector housed in a 6 cm thick lead shield and lined with cadmium and copper sheets. The shield assisted in reduction of the background radiation. The samples were mounted on the detector surface and each counted for 29,000 seconds in producible sample-detector geometry. The configuration and geometry was maintained through the analog. A computer based Multichannel Analyzer (MCA) Maestro programme from ortec was used for data acquisition and analysis of gamma spectra. The 1764 KeV gamma-line of ^{214}Bi was used for ^{238}U in the assessment of the activity concentration of

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^{226}Ra while 2614.5 KeV gamma-line of ^{208}Tl was used for ^{232}Th. The single 1460 KeV gamma-line of ^{40}K was used in its content evaluation. All the obtained raw data were converted to conventional units using calibration factors to determine the activity concentration of ^{40}K, ^{226}Ra and ^{232}Th as presented in Table 1:

Table 1. Energy Calibration for Quantitative Spectra Analysis

Isotopes	$\times 10^{-3}$ (cps/ppm)	$\times 10^{-4}$ (cps/Bq/kg)	Conversion factors (Bq/kg (ppm))	Ppm	Bq/kg
^{40}K	0.026	6.431	0.032	454.54	14.54
^{226}Ra	10.500	8.632	12.200	0.320	3.84
^{232}Th	3.612	8.768	4.120	2.27	9.08

The net number of counts under each photo peak of interest was then background subtracted using the time correct spectrum taken using the blank container. The activity concentration was calculated using Equation 1 [8,9].

$$\text{Activity (Ra, Th and K)} = \frac{count\ rate\ (cpm) for\ Ra,\ Th,\ K}{count\ rate\ (cpm) for\ Ra,\ Th,\ K} \quad (1)$$

2.4 Assessment of Radiation Hazards Associated with the Ingestion of Soil

2.4.1 Radium Equivalent Activity (Ra_{eq})

To represent the activity levels of Ra-226, Th-232 and K-40 by a single quantity, which takes into account the radiation hazards associated with them, a common radiological index called Radium equivalent activity was used. This parameter was calculated using Equation 2 [10,11] based on the assumption that 10 Bq/kg of Ra-226, 7 Bq/kg of Th-232 and 130 Bq/kg of K-40 produce equal gamma dose.

$$R_{eq}\ (Bq/Kg) = C_{Ra} + 1.43C_{Th} + 0.077C_K \quad (2)$$

Where C_{Ra}, C_{Th} and C_K are the activity concentrations of Ra-226, Th-232 and K-40 respectively.

2.4.2 Annual Effective Dose from Ingestion

From the activity concentration of Ra-226, Th-232 and K-40 in the soil samples, the annual effective dose due to the ingestion of soil in humans was estimated using Equation 3 [12,13]

$$E = (U_{Ra}C_{Ra} + U_{Th}C_{Th} + U_kC_k)\ M \quad (3)$$

Where, M is the annual average quantity of soil ingested per person in Nigeria which was adopted as 9.13 kg/capital/year [14]. C is the specific activity concentration of radionuclides in soil determined in this work, and U refers to the effective dose coefficients measured for the radionuclides (Sv/Bq) for different age groups for the ingestion of natural radionuclides ^{226}Ra, ^{232}Th and ^{40}K with values of 4.50×10^{-8}, 2.30×10^{-7} and 6.20×10^{-9} respectively [12-15].

2.4.3 Alpha Index

Alpha Index (I_α) is used to estimate the hazards that could arise from the ingestion of soil. this index is computed using Equation 4 [16-18]. For radiation protection purposes, the value of alpha index must not exceed the limit of unity. The maximum value of I_α equal to unity corresponds to the upper limit of radium equivalent activity 370 Bq.kg^{-1}.

$$I_\alpha = \frac{Cra}{200\left(\frac{Bq}{kg}\right)} \quad (4)$$

2.4.4 Fertility Cancer and Hereditary Risks

The cancer and hereditary risks due to low doses without threshold dose known as stochastic effects were estimated using Equation 5 and 6 respectively based on ICRP, 2007 cancer risk assessment methodology. The lifetime risks (70 years) of fatal cancer were based on the hypothesis of linearity of dose and effect without any threshold. The nominal risk coefficients for low doses as adopted from ICRP based on data for cancer incidence weighted for lethality and life impairment were 5.5×10^{-2} and 0.2×10^{-2} for cancer and hereditary risks, respectively, these values were derived by [19].

Fatality cancer risk = Total AED (Sv) × Cancer Nominal Risk Factor (5)

Hereditary risk = total AED Sv × hereditary nominal risk factor (6)

3. Results and Discussion

The spectra of twelve surface soil samples surrounding the Culombite mine have been analyzed. The specific activity of ^{40}K, ^{226}Ra, ^{232}Th.

Table 2 presents the activity concentration of the naturally occurring radioactive materials in twelve (12) different soil samples. ^{226}Ra had the lowest activity concentration in each sample compared to ^{232}Th and ^{40}K, while ^{40}K had the highest activity concentration in all the samples except "NW3 A" which has lower concentration analyzed as expected since Potassium is an important nutrient for man and is naturally available in abundance. The activity concentration of ^{226}Ra, ^{232}Th and ^{40}K in the twelve (12) different soil samples, varied widely and had an average ± error values of 28.43 ± 5.28 Bq/kg, 66.84 ± 2.02 Bq/kg and 645.29 ± 7.32 Bq/kg respectively. NW3 C was found to have the highest activity concentration of 1026.13 ± 7.62 Bq/kg for ^{40}K while NW2 B was found to have the lowest activity concentration of 268.27 ± 4.51 Bq/kg for ^{40}K. Activity concentration of ^{226}Ra was found to be high-

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est (54.58 ± 8.23 Bq/kg) in NW4 B and lowest (6.49 ± 1.27 Bq/kg) in NW2 A. It was observed that NW4 B had the highest activity concentration values of 83.12±0.46 Bq/kg and the lowest of 42.65±5.25 Bq/kg for ^{232}Th.

Table 2. Specific Activity of the NORMs in the Analyzed Samples

Sample codes	k-40 (Bq/kg)	Ra-226 (Bq/kg)	Th-232 (Bq/kg)
NW1 A	0569.98±09.95	19.35±02.32	79.93±1.03
NW1 B	0536.39±08.55	24.91±00.12	67.50±0.11
NW1 C	0530.48±09.49	33.60±07.02	63.06±1.37
NW2 A	0239.04±05.60	06.49±01.27	52.79±1.77
NW2 B	0268.27±04.51	20.63±05.33	42.65±5.25
NW2 C	0646.19±05.91	35.46±10.78	78.45±4.10
NW3 A	048.52±03.58	44.96±03.71	73.32±0.46
NW3 B	0570.30±06.53	33.60±06.61	65.34±4.79
NW3 C	1026.13±07.62	18.31±00.48	62.71±1.61
NW4 A	0537.48±11.20	37.89±07.88	71.38±2.28
NW4 B	0283.83±08.40	54.58±08.23	83.12±0.46
NW4 C	0551.01±06.53	11.36±09.62	61.80±1.03
Range	268.27-1026.13	6.49-54.58	42.65-83.12
Average	0645.29±07.32	28.43±05.28	66.84±2.02

The radiological parameters associated with the ingestion and inhalation of naturally occurring radioactive materials in soil samples are presented in Table 3. Considering the annual average quantity of soil ingested or inhaled per person in Nigeria as 14 kg/year [20], the average annual effective dose due to the ingestion of soil in humans was estimated at 0.36±0.1μSv/y which was far (approximately 1000 times) lower than the world average annual committed effective dose of 300 μSv/y for ingestion of natural radionuclides provided in [20]. At the present average soil ingestion or inhalation rate of 9.13 kg/year in Nigeria [20], the annual effective dose is far below the acceptable limit, however, people mining in the sample area may have higher ingestion or inhalation rates than that reported by [20]. Consequently, it is important to predict the threshold ingestion or inhalation rate above which the average annual effective dose will exceed the acceptable threshold of 300 μSv/y. Figure 1 presents the average annual effective dose as a function of ingestion or inhalation rates. From the figure it could be observed that for ingestion or inhalation rates between 0 and 40 kg/yr, the AED is within the acceptable limit, therefore the threshold ingestion or inhalation rate is 40 kg/yr and any value slightly higher than the threshold values is prone to significant radiological health risk. In order to safeguard the members of pubic from the radiological hazards associated with the soil ingestion or inhalation, radium equivalent activity Ra_{eq} (Bq/kg), alpha index and total cancer risk were estimated and found to be 161.44±8.08 Bq/kg, 0.142±0.02 and (0.21±0.05) $\times 10^{-5}$ respectively. UNSCEAR stipulated that; radium equivalent activity should not exceed 370 Bq/kg and alpha index should not exceed the limit of unity, annual effective dose due to ingestion or inhalation of naturally occurring radioactive materials in soil, medicinal plants and food should not exceed 300 μSv/y [20], hence the values obtained in this work were within the acceptable limits. Similarly, USEPA stated that the maximum acceptable total cancer risk should not exceed 1 $\times 10^{-4}$, since all the cancer risks obtained in this work were by far (approximately 100 times) less than the acceptable threshold, it implies that the ingestion or inhalation of soil is not associated with any radiological risk of concern. The fatality cancer risk for almost all the samples were found to be approximately 30 to 40 times the hereditary cancer risks. Of all the samples analyzed, NW2 C had the highest radium equivalent activity, annual effective dose due to ingestion and total cancer risk values of 197.40 Bq/kg, 2.20×10^{-4} μSv/y and 1.20×10^{-5} respectively while NW2 A had the lowest values of 100.39 Bq/kg for radium equivalent activity, NW2 B had the lowest values of 1.1×10^{-4} μSv/y and 0.63×10^{-5} for annual effective dose due to ingestion or inhalation and total cancer risk respectively. Ingestion or inhalation of NW4 B was found to be associated with the highest alpha index of 0.273, while ingestion or inhalation of NW2 A was found to have the least values of 0.032 for alpha index. Due to the lack of published literature on the radiological levels of soil samples, the activity concentration of natural radionuclides obtained in this work were compared with that obtained for soil and medicinal plants in and outside Nigeria in Figure 2. It is pertinent to note that; the activity concentration of ^{226}Ra reported in this work was greater than that which was reported in soil [18-20], while that of ^{232}Th and ^{40}K were lower than that reported by [16-19] respectively.

Table 3. Radiological Implications of the Ingestion or Inhalation Soil Samples.

Sample Code	Ra_{eq} (Bq/kg)	I_α	$E \times 10^{-4}$ (μSv/yr)	Cancer Risk		
				Fatality $\times 10^{-5}$	Hereditary $\times 10^{-7}$	Total $\times 10^{-5}$
NW1A	177.54	0.097	2.00	1.10	4.00	1.10
NW1B	162.74	0.125	1.90	1.00	3.80	1.00
NW1C	164.62	0.168	1.80	0.99	3.60	1.00
NW2A	100.39	0.032	1.30	0.72	2.60	0.75
NW2B	102.27	0.103	1.10	0.61	2.20	0.63
NW2C	197.40	0.177	2.20	1.20	4.40	1.20
NW3A	153.54	0.225	1.80	0.99	3.60	1.00
NW3B	170.95	0.168	1.80	0.99	3.60	1.00
NW3C	189.00	0.092	1.90	1.00	3.80	1.00
NW4A	181.35	0.189	1.90	1.00	3.80	1.00
NW4B	195.30	0.273	2.10	1.20	4.20	1.20
NW4C	142.16	0.057	1.60	0.88	3.20	0.91
Range	100.39-197.4	0.032-0.273	1.1-2.2	0.61-1.20	2.2-4.4	0.63-1.20
Mean	161.44±8.08	0.142±0.02	0.36±0.1	0.20±0.05	0.72±0.2	0.21±0.05

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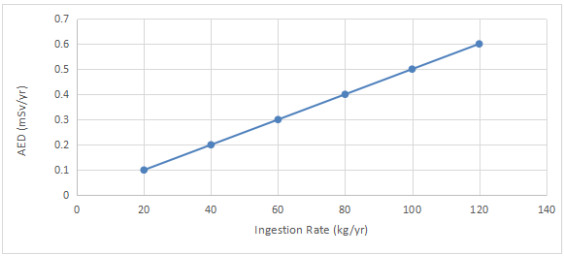

Figure 1. Annual effective dose (AED) due to Soil Ingestion or Inhalation.

It is important to predict the threshold ingestion or inhalation rate above which the average annual effective dose will exceed the acceptable threshold of 300 µSv/yr. Figure 1 presents the average annual effective dose as a function of ingestion or inhalation rates. From the figure it could be observed that for ingestion or inhalation rates between 0 and 40 kg/yr, the AED is within the acceptable limit, therefore the threshold ingestion or inhalation rate is 40 kg/yr and any value slightly higher than the threshold values is prone to significant radiological health risk.

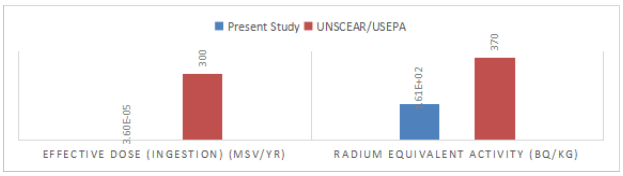

Figure 2. Annual effective dose (AED) and Radium Equivalent Activity (Ra_{eq}) due to Soil Ingestion or Inhalation.

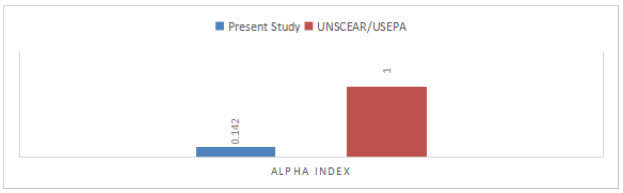

Figure 3. Alpha Index (I_α) due to Soil Ingestion or Inhalation.

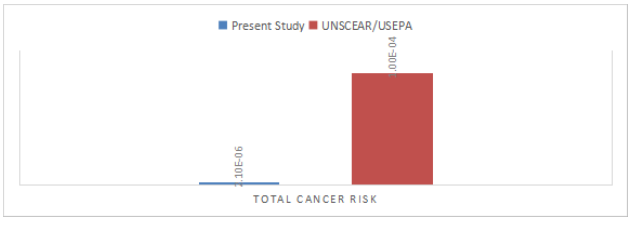

Figure 4. Total Cancer Risk due to Soil Ingestion or Inhalation.

4. Conclusions

At the present average Soil Ingestion or Inhalation rate of 14.4 kg/y in Nigeria, the average annual effective dose due to the Soil Ingestion or Inhalation in humans was approximately 1000 times lower than the world average annual committed effective dose of 300 µSv/yr for ingestion of natural radionuclides provided in UNSCEAR 2000 report. It was established that for Ingestion or Inhalation rates between 0 and 40 kg/y, the AED is within the acceptable limit, therefore the threshold Ingestion or Inhalation rate is 40 kg/y and any value slightly higher than the threshold values will be associated with a significant radiological health risk. The radium equivalent activity (Ra_{eq}) and alpha index were far lower than their UNSCEAR acceptable thresholds of 370 Bq/kg and 1 respectively. Furthermore, the total cancer risk due to fatality and hereditary effects that may arise from Ingestion or Inhalation was approximately 100 times less than the USEPA acceptable threshold of 1×10^{-4}. Among all the soil varieties analyzed, NW2 C had the highest radium equivalent activity (Ra_{eq}), annual effective dose due to ingestion and total cancer risk values while NW2 A had the lowest values of these parameters. Therefore, the present Ingestion or Inhalation rate of soil in the area poses no radiological risk to the population.

Disclosure Statement

No potential conflict of interest was reported by the author(s).

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ARTICLE

Spectrum of Pediatric Malignancies: An Observational Single Center Study from Western India

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ABSTRACT

Cancer is a leading cause of death for children and adolescent worldwide. The cure rates in low middle-income countries are dismal (20%) in comparison to high income countries (80%). The first move is to assemble precise data on epidemiology of pediatric cancer across the country and its region wide variation. This study attempts to provide spectrum of pediatric malignancies from a tertiary care hospital in the state of Rajasthan, India.

A total of 140 cases were studied retrospectively over a period of two years (April 2018-March 2020). Patients, 0-18 years of age that are diagnosed as a case of malignancy were included in this study. The records of these patients were retrieved and analyzed.

Patients were stratified in 4 groups; 0-4 years, 5-9 years, 10-14 years and 15-18 years. Most of the patients fell in 15-18 year group (35.7%), followed by 5-9 year group (28.5%). Majority of cases, 67.8% were male. The male to female ratio is 2.1:1. Leukemia (40%) was the most common malignancy followed by lymphoma, retinoblastoma and malignant bone tumors. Acute lymphoblastic leukemia comprises majority (35/56) of leukemia. Retinoblastoma was predominant malignancy among <5-year children. In all other groups, leukemia was predominant.

This study gauges the trend of pediatric malignancies at one of the largest tertiary care hospitals in Rajasthan, which is important in the planning and evaluation of health strategies. As we lack a dedicated pediatric cancer registry, such epidemiological studies play a significant part for this small but distinguished group of patients.

1. Introduction

Cancer is one of the leading causes of death for children and adolescents around the world and approximately 300,000 children aged 0 to 19 years old are diagnosed with cancer each year [1].

In comparison to world, India has a lower incidence of pediatric cancer. As per the report of International Incidence of childhood cancer volume-3 (IICR-3), age-standardized rate of childhood cancer (0-19 year) incidence in India is 87.3 per million (pm) which is significantly lower than countries like US (180 pm), Canada (173.9 pm), Europe (170-190 pm) [1].

In India, data is collected through 33 population-based cancer registry and 29 hospital-based cancer registry which represents just 10% of population [2,3]. This high-

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lights our knowledge gap regarding the true incidence of pediatric cancer in our country due to under reporting. Hence, leading to less diversion of resources for the management of pediatric cancer care and resulting in dismal outcomes in comparison to the western world [3,4]. Moreover there is regional variation in reporting due to disparity in infrastructure and socioeconomic factors. Our study is an attempt to strengthen the pediatric cancer epidemiological data and emphasize the demographic variations.

2. Material and Methods

It is a retrospective observational cohort study conducted over a period of two years (April 2018 to March 2020) in the department of medical oncology at a government tertiary health care cancer facility of Rajasthan after obtaining permission from concerned authority. The data of total 140 cases were collected from hospital records. All children aged 0-18 years, diagnosed as a case of malignancy by means of peripheral blood smears and bone marrow studies, cytological and histopathological examination during this period, were included in the study. Histological diagnosis was confirmed by our pathologist in all cases except for surgically inaccessible intracranial tumors. The records of these patients were retrieved and analyzed, focusing on the prevalence according to age, sex and types of tumors. For classification of pediatric malignancies in the present study, the International Classification of Childhood Cancers (ICCC), based on International Classification of Diseases for Oncology (ICD-O-3), was followed [5,6].

Statistical analysis

The data were entered in an EXCEL sheet and then analyzed. Descriptive statistics for continuous variables and frequency distribution, with their percentages were calculated as required.

3. Results

The data were recorded for 140 patients from age 0-18 years. Patients were stratified in four groups i.e 0-4 year, 5-9 year, 10-14 year and 15-18 year (Figure 1). Most of the patients (35.7 %) were placed in 15-18 year group (50/140), followed by 28.5% (40/140) patients in 5-9 year group. There were 18.5% and 17.1 % patients from age group 10-14 years (26/140) and 0-4 years (24/140). The mean and median age is 10.3 years and 11 years respectively in the present study. Sex wise distribution: Majority of cases, 67.8% were male (95/140) in comparison to 32.1% (45/140) were female (Figure 2). The male to female ratio is 2.1 in the current study.

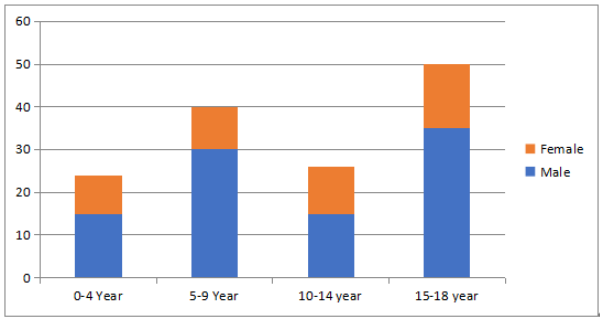

Figure 1. Stratification of patients as per age groups.

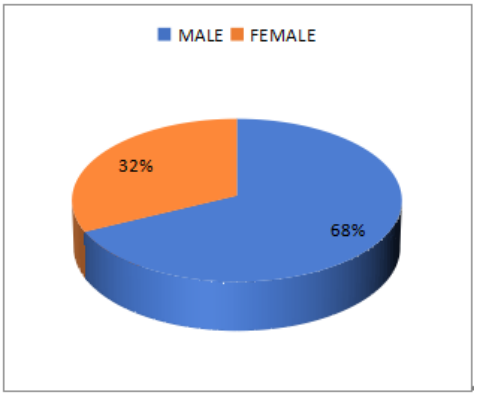

Figure 2. Sex wise distribution

Clinical Profile

Among all pediatric cancers, the most common was leukemia with 40% (56/140) of children affected (Figure 3/Table 1). The second most common was lymphoma 14.2% (20/140), followed by retinoblastoma 11.4% (16/140) and malignant bone tumors 10% (14/140). Germ cell tumor, neuroblastoma and renal tumors each constitute five percent (6/140) cases. Soft tissue sarcoma and CNS neoplasm were 5% (7/140) and 1.4% (2/140) respectively. Among others, there was a case of adrenocortical tumor in a 17-year-old boy.

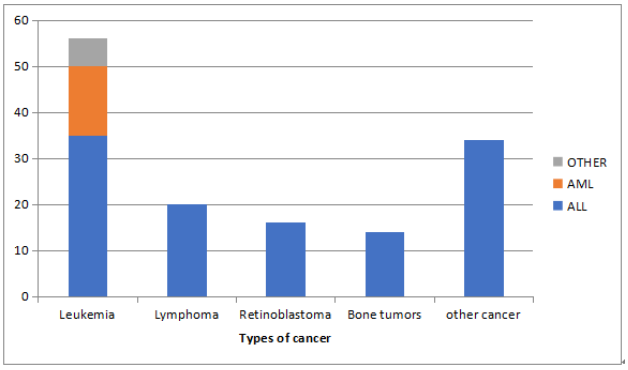

Figure 3. Frequency of various cancers among study population

Table 1. Distribution of various cancers along the age groups

S.no.	Type of cancer		0-4 Years	5-9 years	10-14 years	15-18 years	Total	Total (percentage %)
1.	LEUKEMIA	ALL	0	12	7	16	35	56 (40%)
		AML	1	3	3	8	12	
		CML	0	0	1	3	04	
		MDS	0	0	1	0	01	
		Unspecified	0	0	1	0	01	
2.	LYMPHOMA	Hodgkin lymphoma	0	3	1	6	10	20 (14.2%)
		NHL	2	2	0	2	06	
		OTHER	1	1	2	0	04	
3	CNS NEOPLASMS		1	0	1	0	02	2(1.4%)
4	NEUROBLASTOMA		1	3	0	2	06	6 (4.2%)
5	RETINOBLASTOMA		9	5	1	1	16	16 (11.4%)
6	RENAL TUMORS (Wilms tumor)		3	3	0	0	06	6 (4.2%)
7	HEPATIC TUMORS		0	0	0	0	00	0
8	BONE TUMORS	Osteosarcoma	0	0	1	2	03	14 (10%)
		Ewing sarcoma	1	2	4	3	10	
		Other	0	0	0	1	01	
9	SOFT TISSUE SARCOMA	Rhabdomyosarcoma	1	1	0	1	03	7(5%)
		Other STS	1	2	1	0	04	
10	GERM CELL TUMOR		2	1	0	3	06	6(4.2%)
11	CARCINOMA AND MELANOMA		1	0	0	1	02	2 (1.4%)
12	OTHERS AND UNSPECIFIED		0	2	2	1	05	5 (3.5%)
Total			24	40	26	50	140	140
Percentage			17.1%	28.5%	18.5%	35.7%		100%

Among the subgroup of leukemia, acute lymphoblastic leukemia was the most common with 62.5% (35/56) cases. The most common age group affected was between 15 to 18 years with male predominance. Acute myeloid leukemia was 10.7% (15/140) of all the cases. There were 4 cases of chronic myeloid leukemia, 3 of them lie in age group 15 to 18. There was 10-year-old boy having myelodysplastic syndrome. Among the lymphoma subgroup, Hodgkin lymphoma was the commonest with 7.1% (10/140) cases. There was again male predominance with only single female case out of 10. The most common age group affected was 15 to 18 years. There were 4.2% of non-Hodgkin lymphoma and 4 cases of unspecified lymphoma. Ewings sarcoma (7.1%) was the commonest bone tumor followed by osteosarcoma (2.1%). Most of our patients (80%) were started on treatment protocol as per the diagnosis (Figure 4). Seven percent refused for further treatment and 13% were referred to palliative or best supportive care.

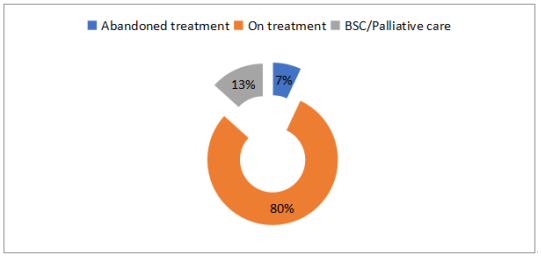

Figure 4. Follow up of the study cohort

4. Discussion

Childhood cancers are often neglected as they represent a small proportion of all cancers (0.7-4.4%) [3,7]. On the other hand when it occurs, it requires medical, psychological and societal concern. Childhood cancer incidence appears to be increasing in India [3,8]. As we contain the morbidity and mortality caused by infection and malnutrition, childhood cancer attain increasing priority in our country.

In the report of International Incidence of childhood cancer volume-3 (IICR-3), age-standardized rate of childhood cancer (0-19 year) incidence in India is 87.3 pm which is quite lower than countries like US (180 pm), Canada (173.9 pm) or Europe (170-190 pm) [1]. This discrepancy can be explained by delay in diagnosis, under-reporting, poor health care access, centralization of resources, less than 10% population coverage by cancer registries. 'Missing' cases can be attributed to myriad of reasons ranging from societal to availability of health care services [3].

In this study, we retrospectively analyzed the data regarding demographics and spectrum of malignancies in 140 pediatric patients (0-18 years) in a span of two years attending our tertiary health care facility.

As per IICR-3 [1], ASRs were higher in children aged 0-4 years (ASR 197.1 pm) and 15-19 years (ASR 185.3 pm) than in those aged 5-9 years and 10-14 years. Similar observation made in our study for the age group 15-18

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years of age (35.7%; 50/140 cases), but not for 0-4 years. The possible explanation can be that this age group of 0-4 years is obtaining treatment at the pediatric centre of our institute.

Incidence rates are slightly higher in boys than in girls (incidence sex ratio 1•14 in the 0-19 years age-group) and varied with age, region, and diagnostic group [1,3]. In IICR-3, the highest sex ratio incidence was reported from India (1.56) compared to 1.12-1.15 in high income countries (3). In our study, males were affected in 65.7% (95/140), while females were affected in 34.3% (45/140) cases. M: F ratio was 2.1:1. Similarly, Jussawalla et al [9] (1.7), Das et al [10] (2), Nandkumar et al [11] (1.8), Chauhan et al [12] (2.2) and Bryan et al [13] (4) reported high sex ratios in their studies. Although according to Kusumakumary et al [14], male predominance is a salient feature of many childhood tumors. This high ratio cannot be explained solely biologically or genetically but a large number of socio-cultural practices play in their part. Gender-based discrimination is seen in Southeast Asian countries which results into delayed healthcare seeking for all childhood illnesses including cancer [3,15].

Childhood cancers are more commonly derived from hematopoietic system, central nervous system, soft tissue, bone and kidney in contrast to adults in whom skin, lung, breast, prostate and colon are the mostly affected [16]. The three most common tumor in our study were leukemia (56/140; 40%), lymphoma (20/140;14.2%) and retinoblastoma (16/140;11.4%). Bhalodia et al. [17], Pattnaik et al. [18], Jan M et al. [19], Chauhan et al [12] and Chaudhuri et al. [20] also reported leukemia as the most common pediatric malignancy in their studies. IICR-3 [1] also reports leukemia as the most common cancer for 0-14 year but lymphoma among 15-19 year. Lymphoma comprises 16% (8/50) of patient among 15-18 year in this study. Our data found retinoblastoma as the most common malignancy among 0-4 year (9/24; 37.5%). Similar findings were reported by Jabeen et al [21] and Hazarika et al [22]. Leukemia was the most common malignancy among all other age groups (37-54%).

Malignant bone tumors were present in 10% (14/140) of our patients. This is in concordance with Pattnaik et al. [18], Chauhan et al. [12] and Devi S et al [23]. As in IICR-3 [1], renal tumors were common in children aged 0-4 years (3/24;12%) and 5-9 year (3/40;7.5%) and frequency decreased in older age groups (0%). ALL was the most commonly seen hematological malignancy (62%; 35/56 cases). This was in concordance with the studies of Bhalodia et al. [17], Satyanarayana et al. [8], Pattnaik et al. [18] and Chauhan et al [12]. Retinoblastoma was the most common non-hematological malignancy (16/64; 25%) followed by Ewing sarcoma (10/64; 15.6%). Chaudhuri et al. [20] also reported retinoblastoma as the most common non-hematological malignancy (19.2%). There was no case of hepatic tumor in our cohort and CNS neoplasm was observed in only 1.4% (2/140) cases. This may be due to delay in diagnosis, poor availability of imaging techniques and their prohibitive cost.

A SIOP report stressed that refusal; non-compliance and abandonment of medical treatment remain critical issue [24]. Although most of the patients (80%) in our study were started on disease-based protocol, further follow up data could not be retrieved. Twenty percent were not given disease specific treatment as few (7%; 10/140) refused and rest (13%; 19/140) had very advanced disease. Arora et al [25] reiterates the problem of abandonment in the developing countries for child hood cancer and suggests ways to improve treatment adherence.

Hence, we notice that various studies have shown inconsistent pattern of childhood cancer from our country. Retinoblastoma and leukemia were the most common malignancy in 0-4 year and 5-18-year group respectively. Leukemia, lymphoma, bone tumor and germ cell tumor occurred more commonly above five years of age, while retinoblastoma and Wilm's tumor were seen mostly in children less than five years.

Limitation

The present study is a single institution-based study. Small sample size and lack of follow-up served as a limitation.

5. Conclusions

This study gauges the trend of pediatric malignancies in Rajasthan, which is important in the planning and evaluation of health strategies. In India, where there is dearth of high-quality data as we lack a dedicated pediatric cancer registry, such epidemiological studies play a significant part for this small but distinguished group of patients.

Conflict of Interest

There was no conflict of interest.

All publication ethics were followed as per COPE guidelines.

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ARTICLE

The Loss of Heterozygosity of *FHIT* Gene in Sporadic Breast Cancer

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ABSTRACT

The loss of heterozygosity (LOH) is a genetic event that can change gene function. *FHIT* is a potential tumor suppressor gene. Although the precise FHIT molecular mechanism of action is not well understood, evidences suggest that Fhit protein reduced levels are involved in mammary carcinogenesis. The aim of this study was to investigate if *FHIT* LOH could influence on sporadic breast cancer (BC) biological behavior, through its association with prognostic factors for sporadic BC.

Tumor tissue and peripheral blood samples were analyzed using the microsatellite marker D3S1300. The findings were associated with clinicopathological parameters including overall survival. LOH was detected in 31.1%(52/167) of the informative BC' cases. Considering clinical and pathological characteristics we have found no significant association with *FHIT* LOH status. The mean follow-up time was 80 months. After the Cox regression analysis two parameters remained associated with BC's risk of death: TNM stage III and IV - HR = 3.74(95% CI, 1.16-12.1) P=0.027 and disease relapse HR = 3.14(CI 95% 1.26-7.80) P =0.014.

This study shows that *FHIT* LOH by itself is not a prognostic factor for sporadic BC. Further researches are required to elucidate the functional role of *FHIT* LOH concerning to BC.

1. Introduction

Breast cancer remains the most common cause of cancer-related deaths in women globally [1].

Innumerous are the efforts to find and understand the set of genetic and epigenetic changes that can influence mammary carcinogenesis and tumor biological behavior [2].

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Over the last years, advanced molecular techniques have brought to the research front opportunities to investigate new biomarkers for BC [2-3].

The *FHIT* gene is located on chromosome 3p14.2, encompassing the most common fragile site of the human genome, the FRA3B. The degree of fragility at this site may provide greater susceptibility to chromosomal rearrangements, allelic losses and breaks: alterations frequently observed in neoplastic cells [4].

Although *FHIT* function is not completely understood, this gene is considered a potential tumor suppressor. It has been involved in the pathogenesis of several tumors, including BC [5].

The loss of heterozygosis (LOH) is a genetic change commonly observed in human cancers. It occurs when a chromosomal region is lost, leading to changes in the gene function [6].

Regarding to BC the role of *FHIT* LOH is still on debate. Ahmadian *et al*. [7] inferred that *FHIT* LOH would be an early but not independent event in BC's carcinogenesis. De Oliveira *et al.* [8] have shown an association between *FHIT* LOH and ductal BC subtype. While Santos *et al*. [9] ÿÿÿÿÿÿÿÿÿÿÿÿÿÿÿÿÿÿ found no connections between BC's clinical features and *FHIT* LOH. Considering the potential of *FHIT* LOH as a prognostic factor for BC, only Ingvarsson *et al.* [10] reported a relationship between *FHIT* LOH and patient's survival.

In this sense, our study aimed to analyze *FHIT* LOH in a cohort of BC's patients and to investigate its possible association with clinical pathological factors and survival.

2. Materials and Methods

2.1 Study Population

The study sample was composed by paired tumor tissue and blood samples from 214 female patients diagnosed with sporadic breast cancer, from 2009 to 2014, at the Mastology Clinic of Femina Hospital, a reference women hospital in Southern Brazil.

Patients with proliferative mammary disorders, history of other types of cancer and male patients were excluded from the study.

According to the study protocol, all patients were evaluated on pre-operatory period to diagnose and stage the disease. Clinical and pathological data were filled in a standardized form. Data regarding to pathological characteristics were collected from pathology reports.

Tumor stage was established according to AJCC (2017).

2.2 Collection of Peripheral Blood and Tumor Tissue

Peripheral blood samples were collected by intravenous puncture. About 10 ml of peripheral blood in sterile tubes containing anticoagulant were collected from each patient.

Samples of the tumor tissue were obtained during the surgical procedure to treat the disease. Tumor samples were prepared for frozen sections, to check if there was sufficient amount of tumor to emit a reliable signal in the LOH analysis.

The blood and tumor tissue were packaged in sterile tubes and stored at -80ºC for later DNA extraction.

2.3 Procedures

Genomic DNA was extracted from the samples: tumor tissue and blood sample, using PureLink™Genomic DNA Mini Kit (Invitrogen™, Carlsbad, CA, USA), following manufacturer's instructions (Invitrogen Kit Handbook). In all the samples, the quality measurement of the extraction product was determined by spectrophotometry (NanoDrop).

Polymerase chain reaction (PCR) was used to amplify the microsatellite marker D3S1300, located within FHIT gene (intron 5). The primers were constructed according to the information of reference [11] AGCTCACATTCTAGTCAGCCT/GCCAATTCCCCAGATG and forward primer was labeled fluorescent dye 6-FAM.

The reactions were prepared to a final volume of 15 uL, containing 0.5 uL of genomic DNA, PCR buffer 1X, 1.5 mM $MgCl_2$, 200 µM of dNTPs, 750 nM of each pair of primer and 1,0 unit of platinum *Taq* DNA polymerase (Invitrogen ™, Carlsbad, CA, USA). Each microsatellite was amplified using genomic DNA (tumoral tissue and blood samples), after initial denaturation during 5 minutes at 96°C, were realized 35 cycles of the following stages: denaturation at 96°C for 30 seconds, annealing ate 55°C for 30 seconds and extension at 72°C for 1 minutes, followed by a final extension at 72°C for 20 minutes.

2.4 LOH Analysis

To verify its specificity and approximate concentration, the PCR products were analyzed by electrophoresis in agarose 1% gel stained with ethidium bromide and visualized under UV translluminator.

PCR product (1 µL) was added and homogenized with to 8.5 µL of formamide HiDi (Applied Biosystems, Foster City, CA, USA) and 0.5 of GeneScan 500 LIZ™ (Applied Biosystems, Foster City, CA, USA). Samples were denaturized for 5 minutes at 95°C and subsequently evaluated by capillary electrophoresis on ABI-PRISM 3130 (Applied Biosystems™, Foster City, CA, USA). The results were analyzed by the software GeneMapper™, version 4.0 (Applied Biosystems™, Foster City, CA, USA).

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Table 1. Cox regression analysis for overall mortality.

Variables	Univariate analysis			Multivariate analysis[a-b]	
	HR (CI 95%)	*P*		HR (CI 95%)	*P*
LOH	1.40 (0.67-2.92)	<0.360			
Age, years	1.03 (0.99-1.07)	<0.067		1.01 (0.98-1.05)	<0.216
Tumour size, cm	1.22 (1.07-1.39)	<0.002		1.10 (0.96-1.26)	<0.162
Tumour grade		<0.169			
II	1.39 (0.32-5.92)				
III	2.12 (0.49-9.05)				
Histological type, lobular	1.33 (0.47-3.70)	<0.582			
TNM stage, III-IV	9.06 (3.78-21.6)	<0.001		3.74 (1.16-12.1)	<0.027
Vascular invasion	3.18 (1.41-7.15)	<0.005		1.03 (0.38-2.80)	<0.942
Molecular phenotype		<0.012			<0.647
Luminal B	2.39 (0.81-6.98)			1.58 (0.36-6.77)	
HER2	6.23 (1.68-23.1)			2.57 (0.44-14.7)	
Triple negative	3.70 (1.13-12.1)			2.21 (0.60-8.11)	
Estrogenic receptor, positive	0.41 (0.20-0.84)	<0.015		0.85 (0.17-4.23)	0.852
Ki67, >14%	1.53 (0.73-3.23)	<0.256			
Neoadjuvant chemotherapy	1.58 (0.79-3.14)	<0.193			
Disease relapse	7.88 (3.87-16.0)	<0.001		3.14 (1.26-7.80)	<0.014

Abbreviations: FHIT, fragile histidine triad; HR, hazard ratio; LOH, loss of heterozygosis. CI, confidence interval.

[a] Included in a multivariate model variables p value was less than 0.1.

[b] HR adjusted for age, tumour size, TNM stage, vascular invasion, molecular phenotype, estrogenic receptor and disease relapse.

LOH was calculated as described by Van Houten *et al.* [12]. We considered LOH when we had a ratio score <0.67 or >1.35. Homozygote cases, or those with unclear results, due stutter or artifacts, were considered as non-informative (NI). Values between 0.67 and 1.35 were considered normal heterozygotes.

2.5 Statistical Analysis

Data were presented as frequency and percentage or mean ± standard deviation (SD). We performed associations between variables with the Fisher's exact test. For comparing continuous variables, a Student *t* test or an unequal variance *t* test was used. Kaplan-Meier curves for cumulative survival were compared using log rank test. Survival analysis was performed by Cox proportional hazard regression models: (i) events were defined as the time to death; (ii) censored data were used when the event did not occur at the end of the follow up period. All parameters associated with death (*P* less than 0.1) in univariate analysis were included in a multivariate model and considered statistically significant if the overall *P* value was less than 0.05. Models were adjusted all variables. The analysis supported the assumption of proportional hazard. Data were analyzed using Stata software, version 13 (StataCorp, College Station, TX, USA).

3. Results

From the total of 214 enrolled patients, 47 (22.0%) were homozygotes, non-informative (NI) cases, being excluded from the study. *FHIT* LOH was found in 52 (31.1%) of 167 heterozygotes, informative cases.

Considering clinical and pathological characteristics, *FHIT* LOH status did not present significant association with age, tumor size and grade, TNM stage, vascular invasion, estrogenic receptors status, Ki67 index, molecular phenotype and neoadjuvant chemotherapy (supplementary table).

Regarding the survival analysis, the mean follow-up time was 80 months (min. 4 - max. 96 months). During this time 33 (19.8%) patients died from BC. Since both groups (*FHIT* LOH present and absent) had homogenous clinical data, we performed a Cox regression analysis in order to investigate the role of *FHIT* LOH on patients' survival. After the multivariate analysis, only two parameters remained associated with risk of death: TNM stage III and IV – HR = 3.74 (CI 95% 1.16-12.1) P = 0.027 and disease relapse HR = 3.14 (CI 95% 1.26-7.80) P = 0.014 denoted in Table 1. Thus, as we can see in the Kaplan-Meier curve, *FHIT* LOH had no impact on patients' overall survival (Figure 1).

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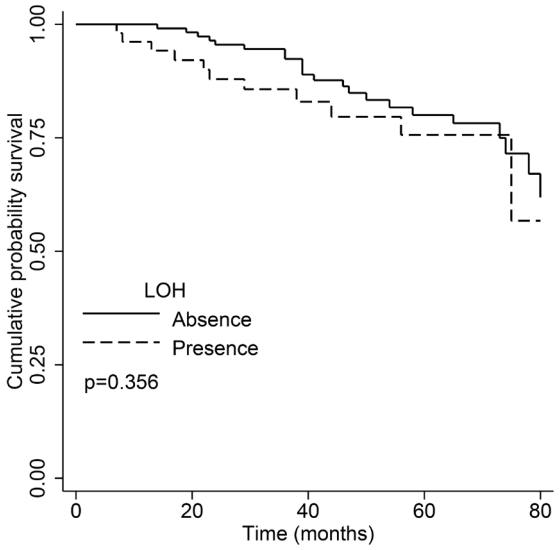

Figure 1. *FHIT* LOH status and patients' overall survival time.

4. Discussion

The precise molecular action mechanism of *FHIT* gene is still unclear, but progress is focused on understanding *FHIT* function related to tumor suppression [13-14].

Studies have pointed out that low levels of Fhit protein could be related with unfavorable parameters in BC [15-17].

LOH at the *FHIT* gene appears to disrupt its function, leading to a reduction in the gene transcripts [7,17].

In the present study, we intended to investigate if the frequency of *FHIT* LOH could influence on tumor biological behavior, through its association with prognostic and predictive factors in sporadic BC's cases.

In our study, the frequency of LOH at the *FHIT* gene detected by the intragenic marker D3S1300 was 31.1% (52 out of 167 informative cases). According to previous published data, *FHIT* LOH frequencies range from 24% to 45%, considering different BC's populations [8-9,11,18-20].

We decided to use the marker D3S1300 because it was able to detect high levels of *FHIT* LOH in prior studies [8-9,11,18,20].

Considering the clinical and pathological variables studied we found no significant association between them and *FHIT* LOH status. Similar results were also reported by Man *et al.* [19] studying low grade BC. Despite the LOH frequency of 40% detected by the marker D3S1300, no association was observed regarding age, tumor size, lymph node stage, presence of vascular invasion, menopausal status, and estrogenic receptor status. Also Santos *et al.* [9] showed no correlation of *FHIT* LOH and tumor size and grade, and axillary metastases. However, the authors highlighted the fact that from the six cases with LOH at the intragenic marker D3S1300, four had also *BRCA1* LOH and suggested that the losses at these genes could be correlated. Subsequently the same group of researchers analyzed 3 markers for each gene LOH: *FHIT* and *BRCA1* in BC's patients and found significant clinical and prognostic association only in the cases with concomitant LOH in both genes. When the LOH was observed exclusively in one of the two genes it had none clinical impact [20]. The idea of simultaneous LOH at distinct chromosome sites is not new. It was demonstrated by Ingvarsson *et al.* [10] an association of *FHIT* LOH and the presence of LOH at other 12 chromosome regions. We should also keep in mind that *FHIT* is located in an active fragile site, the FRA3B. Because of its genomic instability, genetic alterations in other genomic sites could influence on it, predisposing to losses and breaks [14,16,21]. It is also in consonance with the concept of multistep carcinogenesis, in which cancer results from an accumulation of mutations [22].

Few studies analyzed *FHIT* locus changes and patient survival. Some of these use distinctive methodologies and included various tumors types, such as cervical, lung, colon, and gastric cancers [23-25]. When it comes to the LOH at *FHIT* gene and BC´s patients' survival, studies are restricted to two.

Silva Soares *et al.* [20] have shown reduced survival time after 48 months of follow-up of 72 BC's patients who have concurrent *BRCA1* and *FHIT* LOH (P=0.04). Patients with *FHIT* LOH alone had no differences regarding to survival time. The other study including patients' survival analysis enrolled 239 women with BC. After a mean follow-up time of 5 years the authors reported a relative risk of dying for patients with *FHIT* LOH of 1.6 (95% CI: 1.0-2.6) P=0.45. [10] However, despite the effect of *FHIT* LOH on patients' survival, the study sample was composed by women with sporadic and familial BC. From the total of 76 cases with *FHIT* LOH, 30 cases were carriers of BRCA2 999de15 germ line mutation. And besides, it was observed a significant correlation between *FHIT* LOH and ER and PR negativity, which are also parameters associated to familial BC. Our study included 167 informative cases. We intend to analyze if the LOH at the *FHIT* gene alone could impact on clinical pathologic characteristics and patients survival, considering a large sample of exclusively sporadic BC. After multivariate analysis our results evidenced nothing but two well-established independent prognostic factors for BC: TNM stage and disease relapse. The *FHIT* LOH had no significant association with patients' prognoses. This could be due to the fact that we used only one microsatellite marker. Nevertheless we have chosen an accurate marker according to previous studies and the frequency of 31% of *FHIT*

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LOH we detected is within the range published before. According to our data, LOH at the *FHIT* gene is not related to clinical parameters for BC. So we can infer that other mechanisms could explain the association between low levels of Fhit protein and adverse biological behavior demonstrated in earlier researches [15-17,26].

Yang *et al.* [27] have tested the two-hit theory for *FHIT* complete inactivation and found a high index of concordance among LOH (first-hit), promoter hypermethylation (second-hit) and absent Fhit protein expression in BC samples. The authors reported that the *FHIT* hypermethylation was an event much more frequent than LOH in BC. These findings empower the model of biallelic inactivation requirement for the whole silencing of a tumor suppressor gene [28]. And, at the same time, it raises the hypothesis that demethylating-therapy could play a role for such tumors.

Other possibilities cannot be ruled out, as splicing abnormalities [13,29] and coexistent allelic losses at chromosome 3p. [30] Also *FHIT* point mutations, though rarely can occur, and influence on the gene transcripts [7].

Knowledge about the *FHIT* function and role in breast tumors are still not fully explained. Considering that alterations in Fhit protein may have implications in mammary carcinogenesis, further studies are needed to explore the mechanisms whereby these changes occur.

5. Conclusions

Our results show that *FHIT* LOH by itself is not a prognostic factor for BC. However efforts must remain to elucidate the functional significance of LOH at *FHIT* gene and its connection to BC.

The variable frequencies of LOH found in other studies can be explained by some factors, such as different methodologies in which other studies evaluate LOH in samples in paraffin blocks. In different types of samplings, such as sporadic breast cancer, hereditary breast cancer, bilateral breast cancer, benign breast disorders compared to malignant. Another relevant factor is that some studies have evaluated other microsatellite markers that may have been a limitation of our study. We also hypothesized that different results could be found if the study population were hereditary breast cancer. As a future prospect we intend to evaluate *FHIT* gene methylation and protein expression.

Ethics Approval

All the patients were informed about the study and signed the Consent Form, approved with the project, by the Committee of Ethics on Research (Protocol number: 10-641).

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Table

Clinical and pathological characteristics and LOH status of the sample.

Variables	Total (N=167)	LOH		*P*
		Absence (n=115)	Presence (n=52)	
Age, years	55.95±11.96	56.24±11.42	55.29±13.170	<0.586
Tumour size, cm	2.93±2.24	2.69±1.83	3.48±2.910	<0.220
Tumour grade				<0.808
I	18 (10.8)	13 (11.3)	05 (9.6)0	
II	83 (49.7)	55 (47.8)	28 (53.8)	
III	66 (39.5)	47 (40.9)	19 (36.5)	
TNM stage				<0.053
I-II	109 (65.3)0	81 (70.4)	028 (53.8)	
III-IV	58 (34.7)	34 (29.6)	024 (46.2)	
Vascular invasion				<0.228
No	77 (51.3)	55 (55.0)	22 (44.0)	
Yes	73 (48.7)	45 (45.0)	28 (56.0)	
Molecular phenotype				<0.988
Luminal A	51 (31.1)	31 (27.4)	20 (39.2)	
Luminal B	73 (44.5)	57 (50.4)	16 (31.4)	
HER2	10 (6.1)0	06 (5.3)0	04 (7.8)0	
Triple negative	30 (18.3)	19 (16.8)	11 (21.6)	
Estrogenic receptor				0.333
Negative	40 (24.0)	25 (21.7)	15 (28.8)	<
Positive	127 (76.0)0	90 (78.3)	37 (71.2)	
Ki67				<0.220
14%<	68 (43.9)	43 (40.2)	25 (52.1)	
>14%	87 (56.1)	64 (59.8)	23 (47.9)	
Neoadjuvant chemotherapy				<0.715
No	117 (70.1)0	82 (71.3)	35 (67.3)	
Yes	50 (29.9)	33 (28.7)	17 (32.7)	

Note: Data were presented as No. (%) or mean ± SD.

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REVIEW

Role of Radiotherapy in the Management of Pancreatic Adenocarcinoma: Debate and Discordance in Clinical Trials

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ABSTRACT

Pancreatic adenocarcinoma (PAC) is an extremely fatal malignancy with dismal outcome with standard treatment till date. Investigators are constantly in search of optimal treatment approach and radiation therapy (RT) remains in the centre of debate. Human pancreatic cancer cell lines have shown both intrinsic and hypoxia induced radio resistance, and RT has produced conflicting results as well in the various clinical trials. However, most of the American studies continued the use of RT as a potential treatment modality but the European school of thought is widely criticized for their 'therapeutic nihilism' towards radiation and faulty clinical trial designs.

This article has reviewed the available literature on the evolving role of RT for the management of resectable and borderline resectable PAC and has highlighted the increasing trend towards the use of radiotherapy in both adjuvant and neo adjuvant settings. With the advent of modern RT techniques, the acute and late toxicities are much less than the earlier time, and therefore augmented RT is expected to produce better clinical outcomes for the patients with pancreatic carcinoma.

1. Introduction

Pancreatic adenocarcinoma (PAC) is a formidable gastrointestinal malignancy with nearly 0.49 million of new cases globally in 2020 with staggering number of deaths of 0.46 million patients [1]. It is the 7th most common cause of cancer related death and the incidence and mortality both are much higher in countries with high human development indexes [2]. Smoking, consumption of alcohol, obesity, hypercholesterolemia, diabetes are attributed as modifiable risk factors for PAC [3,4].

In a systematic analysis for the global burden of disease, Pourshams A et al. analysed dataset of 195 countries from 1990 to 2017 and found the incidence and mortality rates of PAC increased in almost all countries over the time and it is alarmingly associated with a substantial number of years of life lost [5]. Although there is a wide geographical variation, this study reported the disability-adjusted life years as nearly 9.1 million globally in 2017. Moreover, using The Surveillance, Epidemiology and End Results stat database, PAC is projected to become second cancer related death by 2030 in the United States [6].

This devastating rate of mortality and cancer burden has kept the investigators desperately motivated in search of the most effective treatment sequence for PAC and to explore multiagent chemotherapy (CT) regimen and chemoradiotherapy (CRT) as both neoadjuvant and ad-

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juvant settings [7,8]. In spite of that, no paradigm shifting treatment option is being established with affirmation in the span of nearly last 50 years and clinical outcome for PAC remain dismal. While radical surgery and chemotherapy are the main treatment options with curative intent, radiation therapy (RT) still remains in the centre of debate in the treatment flowchart. Conflicting data from the published clinical trials which mostly included radiotherapy with earlier techniques and obsolete dose prescriptions is the key reason behind the less acceptance of RT as a potential treatment modality.

This article has reviewed the available literature on the evolving role of RT in the management of resectable and borderline resectable PAC and has highlighted the conflicting data of the clinical trials; however there is a trend towards the increased use of radiotherapy in both adjuvant and neoadjuvant settings for the patients with PAC with excellent clinical outcome.

2. Relative Radioresistance of Pancreatic Cancer Cells

Human pancreatic cancer cells are historically considered as less responsive to external beam radiotherapy, possibly for intrinsic and hypoxia-induced radioresistance. In 1976, Courtenay et al. reported hypoxic fraction as 25% for xenografted pancreatic cancer cells, which indicates the presence of fairly large volume of hypoxic cells [9,10]. In later year, Verovski et al. investigated a panel of eight human pancreatic cell lines and mean inactivation dose was reported as high as for intrinsically radioresistant tumors like melanoma and glioblastoma [11]. In this context, the role of several hypoxic cell sensitizers, such as doranidazole, curcumin, capecitabine are being investigated both clinically and in vitro for pancreatic carcinoma [12,13].

3. Surgery is the Mainstay of Treatment

Pancreaticoduodenectomy (Whipple procedure) followed by adjuvant chemotherapy is considered as the standard of care for resectable PAC, but majority of the disease are either unresectable or borderline resectable at diagnosis. A large number of patients with apparently local disease on imaging already might have occult metastatic disease as well. Moreover, there are high proportions of local recurrences and margin positive surgical resections (R1/R2) after Whipple procedure. As a result of these worse prognostic factors, 10-year overall survival (OS) remain less than 4% for this fatal disease, even after potentially curative resection [14].

4. Evolution of Clinical Trials Involving CRT

Way back in 1958, the regression of tumor was first reported to get enhanced with addition of 5-fluorouracil (5-FU) to RT in an animal model [15]. Upon this principle of synergistic effect of 5-FU, particularly for gastrointestinal tumors, a pilot study was undertaken for the patients with locally advanced or unresectable adenocarcinoma stomach, pancreas and large bowel [16]. Each patient was treated with 900-1200 rads per week to a total tumour dose of 3500-4000 rads, 6 fractions each week along with either 5-FU or placebo. RT portal was planned to encompass the entire clinical target volume but not larger than 20 cm x 20 cm. Survival benefit was noted for all subsets with strikingly better outcome for gastric and pancreatic carcinoma.

4.1 Gastrointestinal Tumor Study Group (GITSG)

The first multicentre, randomized control trial to assess the effect of adjuvant CRT was initiated by GITSG in the United States between 1974 to 1982 [17]. This study was stopped early due to poor accrual, however, it showed a longer median survival (21.0 months vs. 10.9 months; $p < 0.05$) and better 2-year survival (43% vs. 19%) in the group treated with adjuvant CRT. An additional thirty patients were later enrolled to adjuvant CRT arm and the result still confirmed the survival benefit seen in the original study. Based on such encouraging findings, use of adjuvant CRT for PAC was started particularly in the United States.

4.2 Inferior Result with CRT in European Organisation for Research and Treatment of Cancer (EORTC) & European Study Group for Pancreatic Cancer-1 (ESPAC-1) trial

To validate the prior results of GITSG, EORTC started randomization of 218 patients with pancreatic head carcinoma and periampullary carcinoma (between 1987 and 1995) into two groups: adjuvant CRT versus observation alone after surgery [18]. RT was delivered as 40 Gy in a split-dose schedule with concurrent continuous infusional 5-FU. No further maintenance chemotherapy was administered. Median survival and OS in adjuvant CRT arm failed to achieve any statistically significant difference.

Subsequently ESPAC-1 Trial was initiated in 11 European countries in 1994 and randomized 289 patients with resected pancreatic ductal carcinoma into 4 arms by 2x2 factorial design: CRT (n=73) or CT (n=75) neither treatment (n=69), or both treatments (n=72) [19]. Nearly half of the patient population had regional node positive disease, whereas positive margin and local invasion found during surgery were reported as 18% and 20 % respectively. RT was delivered in 40 Gy/split dose schedule along with

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intravenous bolus of 5-FU in first three days of radiotherapy. CT consisted of an intravenous bolus of leucovorin, followed by an intravenous bolus of 5-FU for 5 consecutive days for six cycles. This study found adjuvant CT to produce a significant survival benefit in patients with resected PAC, whereas CRT had a deleterious effect on survival. The estimated five-year survival rate was 10 % in CRT arm, however it was 20 % among patients who did not receive CRT (P=0.05). Taken at face value, results of EORTC and ESPAC-1 study uphold the notion that adjuvant CRT should not be administered routinely for potentially resected PAC.

Counteract the inferior results

Inferior results with the administration of adjuvant CRT as demonstrated by EORTC and ESPAC-1 should be interpreted with caution and subsequent 'therapeutic nihilism' about radiotherapy should be addressed keeping the following factors in mind [20]:

a) These trials including GITSG, EORTC and ESPAC-1 used a low total dose of RT in an obsolete dose schedule. Split dose fractions are radiobiologically inferior because of the accelerated repopulation of tumor clonogens and is no longer used in current practice.

b) Non conformal techniques (AP-PA fashion) for abdominal RT would invariably result into high treatment-related toxicity and decreased survival of the patients.

c) No details of quality assurance (QA) for RT are available. Surgery, or pathological findings are not documented thoroughly.

d) Trials are underpowered and some of them included heterogeneous tumor sites. Clinical outcomes of periampulary carcinoma would be better than PAC and this might influence the overall analysis.

e) More than 20% of patients in CRT arm did not receive the intended treatment because of postoperative complications or lack of compliance in EORTC trial. Not receiving maintenance chemotherapy, unlike GITSG study might be another reason of inferior result with CRT in this particular trial.

Inferior outcome of ESPAC-1 trial has led to subsequent omission of adjuvant RT from most of the adjuvant trials in Europe, including ESPAC-3 and ESPAC-4 [21,22]. The publication of CONKO-001 (Charité Onkologie 001) trial, which was conducted from 1998 to 2004 in Germany and Austria further reduced the practice of adjuvant RT for the patients with locally advanced PAC and adjuvant gemcitabine without RT became the standard of treatment [23].

4.3 Continuing Use of CRT in the United States

American studies involving the management of PAC remained inquisitive regarding the role of radiation and GITSG trial laid the foundation for continuing use of CRT in the United States.

At Mayo clinic in Rochester, 472 patients with PAC were evaluated retrospectively who underwent R0 resection between 1975 and 2005 and 274 patients received adjuvant RT [24]. 45 Gy in 25 fractions was delivered to the tumor bed and regional nodes with a four-field technique followed by an additional 5.4 to 9 Gy boost to the tumor bed. This study reported survival benefit with adjuvant CRT (Median survival 25.2 versus 19.2 months, p=.001). Positive LN and high histologic grade were identified as adverse prognostic factors.

Despite the heterogeneous results of the randomized phase III trials, several nonrandomized, single institute US series have consistently demonstrated survival benefit with the addition of adjuvant CRT for resected pancreatic cancer. Studies conducted at Johns Hopkins University, and an analyses of the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) database are the examples to be mentioned with special emphasis [25,26].

In this context, Radiation Therapy Oncology Group (RTOG)/Gastrointestinal Intergroup trial 9704 was designed to compare 5-FU versus gemcitabine based CT, 3 weeks prior to CRT and 12 weeks after CRT [17,27]. CRT in both arms consisted of 50.4 Gy delivered in 28 fractions (5 days per week) with continuous 5-FU infusion (250 mg/m2/d). RT was delivered to the tumor bed and regional nodes, defined by preoperative CT imaging. Regional nodal stations particularly pancreatic, celiac, mesenteric, periaortic, duodenal, and hepatic portal lymph nodes were included in the RT fields. After an initial dose of 45 Gy, a boost dose of 5.4 Gy was delivered to the tumor bed only. This study included CRT in the both treatment arms and therefore, the independent effect of CRT cannot be assessed. However, it is the largest randomised clinical trial (RCT) that used CRT in the adjuvant settings affirming its contributing role in the management of PAC. Survival benefit at 3 years was demonstrated with the use gemcitabine, but it got disappeared on 5 years of follow up and a large percentage of distant relapse (73%) were reported [28]. Hence, any improvement in survival associated with the use of gemcitabine appears to be temporary and marginal.

These findings prompted the investigators to design a further phase III adjuvant trial to evaluate the impact of CRT after completion of a full course of gemcitabine (NRG Oncology/RTOG 0848) [29]. In the first randomization the impact of the addition of erlotinib to gemcitabine is being tested. After 5 cycles of gemcitabine based therapy, if no evidence of disease progression is found on imaging,

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a second randomization evaluating the impact of CRT would take place. Notable point for this trial is inclusion of 16% of patient population with histologically positive margins. Result of step 1 indicates addition of erlotinib to gemcitabine did not provide survival benefit and the answer regarding the role of adjuvant RT is still awaited.

4.4 Role of Neo Adjuvant RT

Neoadjuvant therapy is believed to produce potential advantages over upfront surgery in patients with localized PAC. With the increased possibility of R0 resection, this approach may lead to a better survival rate and is becoming more acceptable alternative over the years. Furthermore, a vast majority of the patients fail to recover sufficiently or in time after the morbid and extensive surgery leading to the omission or delay of the adjuvant treatment [30]. A meta analysis of 38 studies with the resectable or borderline resectable pancreatic cancer patients reported improved OS by intention to treat with neoadjuvant therapy, despite a drop in the resection rate [31].

The Dutch PREOPANC-1 trial compared neoadjuvant gemcitabine-based CRT to upfront surgery, followed by adjuvant gemcitabine in the both arms [32]. Although OS benefit was not demonstrated, all secondary outcomes found superiority in neoadjuvant arm. Rate of R0 resection, disease free survival, and locoregional recurrence free interval were significantly better with neoadjuvant CRT [33].

With the wide introduction of FOLFIRINOX (5-FU, leucovorin, irinotecan, and oxaliplatin) in the subsequent years as a superior multiagent chemotherapy, PREOPANC-2 trial is further designed with the aim of direct comparison between total neoadjuvant FOLFIRINOX and gemcitabine based CRT [34]. The trial is actively recruiting at present and the result will definitely guide us in future to choose the best neoadjuvant protocol for resectable and borderline resectable PAC.

4.5 Role of Stereotactic Body Radiation Therapy (SBRT)

Role of SBRT is emerging for the management of PAC, since the outcome of conventional CRT is considered suboptimal. With the administration of high dose RT with extreme conformity, the effect of radiation is certainly being augmented and a plethora of clinical trials has demonstrated excellent local control with minimal acute and late toxicity [35-37]. However; the detail discussion of technical feasibility and outcome of SBRT is beyond the scope of this review.

5. Conclusions

Combined modality of treatment is now the accepted rule for the management of PAC, and the role of radiotherapy is being constantly evaluated to get optimally fit into the treatment algorithm. While the margin positive resection and pathologically positive lymph nodes are widely accepted indications for adjuvant CRT, routine use of adjuvant RT is debatable. A total dose of 45-54 Gy to the tumor bed in conventional fractionation schedule is usually accepted, but the elective nodal irradiation and the inclusion of anastomotic sites are not universally followed.

RT has demonstrated adequate efficacy to control pain and obstructive symptoms by shrinking local disease and facilitates R0 resection if administered in neoadjuvant settings. SBRT to a total dose of 30-45 Gy in 3 to 5 fractions can produce excellent local control and presently its role is under evaluation by a plethora of clinical trials. At the time of writing this article, a database of more than hundred clinical trials of SBRT in pancreatic cancer is showing with the search in https://clinicaltrials.gov/ [38]. However, extreme caution need to be taken to minimise the dose to the OARs and respiratory motion management is the another challenge to be dealt with modern radiotherapy techniques.

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REVIEW

Paracelsus Paradox and Drug Repurposing for Cancer

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ABSTRACT

Dose is one of the parameters that any pharmacologist seriously considers when studying the effects of a drug. If the necessary dose to achieve a desired pharmacological effect is in a toxic or very toxic range for human use, the drug will probably fall out from further research. The concentration that a drug can reach to its target organ or cell is a direct consequence of the administered dose and its pharmacodynamic properties. Basic researchers investigate at the cellular level or eventually with xenografts. They use different concentrations of the drug in order to determine its cellular effects. However, in many cases, these concentrations require doses that are in the toxic range or well beyond any clinically achievable level. Therefore, in these cases, research is in the realm of toxicology rather than therapeutics. This paper will show some examples about this exercise in futility which is time and resource consuming but that pullulates the pages of many prestigious journals. Many seasoned researchers seem to have forgotten the Paracelsus Paradox.

1. Introduction

If a cell whether normal or malignant is cultured in distilled water or even in tap water, the cell will die in a short time. Osmolarity in the first place and lack of nutrients will be the cause of death. Therefore, we can say that pure water is cytotoxic for both normal and malignant cells. Now let's change the circumstances, We give a glass of pure water to drink to a normal or sick individual and we shall discover that it is neither bad nor good. It has no effect and it is not cytotoxic. Finally in a new dramatic change of circumstances we administer the same pure water, but this time two liters via the trachea and the individual will be dead in a few minutes. In the movies, this last case would not be considered cytotoxicity but rather death by drowning according to the district attorney. In all three cases the substance used was plain water. In two out of three it was deadly, in one nothing happened.

What kind of a drug is plain water: cytotoxic, neutral or deadly? What changed in each occasion were the circumstances: applied to the cell, received per os, and finally a huge amount received by an unusual administration route.

This little game seems ridiculous, poorly planned and undoubtedly absurd. However, this is the way a large proportion of scientific papers work.

When we treat a malignant cell with 1,000 fold the highest level statins can achieve in circulation and we reach the conclusion that statins can be used as cytotoxic for tumors, aren't we playing the same game as above?

We coined a name for the game: PPP. It is not the Pentose Phosphate Pathway but the Potentiated Paracelsus Paradox.

2. Paracelsus Paradox

Sometime in the 1500s Paracelsus, the Swiss alche-

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mist and physician, established that any curative drug can at the same time be a poison, depending on the dose. We added the Potentiated by modifying one item in the Paracelsus Paradox: any curative drug is at the same time a poison, depending on the dose and the administration route.

Of course, nobody remembers Paracelsus anymore because he committed three sins:

He lived 500 years ago, did not have a Facebook page, and last but not the least, wrote in vulgata, sort of a bad Latin, today's equivalent of the most popular language in science, bad English.

PPP may be a good game for children, but does it have anything to do with the twenty-first century hard science?

If we are supposedly scientist let's examine the evidence before arriving at any conclusions.

3. Statins

We shall start by examining statins. There are many publications maintaining that statins have an anti-tumoral effect. Most of these papers are based on *in vitro* studies. However, these experiments may be misleading, because excessively high concentrations of statins were used, which cannot be reached in patients or if they are reached it would be at the expense of serious toxicity [1]. For example:

►Simvastatin concentration in plasma is in the mean range of 2.2-4.3 nM after an oral administration of 40 mg. The maximum concentration that can be achieved is 19-31 nM [2]. In a publication, *in vitro*, simvastatin at 20 μM induced breast cancer cell apoptosis [3]. This level is almost 1,000-times higher than the maximum that can be achieved in patients. The achievable plasma concentration of simvastatin was published in 2009 (probably there are prior determinations too). The researchers who used a 1,000 higher concentration published their results in 2012. This means that during all their experimental period they knew perfectly well that they were working in the realm of fantasy. A few questions are unavoidable:

Did the peer review process make no objections?

Have the 93 citations of this article in nine years taken for granted that simvastatin is a tumor apoptogenic drug without any further doubt?

Isn't using 1,000 fold the maximum achievable level of the drug like administering four liters of tap water through the trachea?

May be PPP is not a fantasy game but part of a game researchers like to play.

► Similarly, Hoque et al. [4] found that statins were able to induce apoptosis in prostate cancer cells when they were exposed to lovastatin at a concentration of 2 μM. However, a dose of 80 mg reaches a maximum concentration of 50 nM with an average of 9.4 nM [5]. The concentration used by Hoque et al. was 400-fold grater than what can be achieved in a patient. To this we must add that statin concentration in tissues, with the exception of the liver, are much lower than in serum [6]. Thus, most of these effects seen in vitro, are with concentrations many fold higher than those achievable in patients. Part of the game?

► Zhuang et al. [7] investigated the effects of lowering lipid rafts' cholesterol with simvastatin. They found that it reduced PI3K/Akt pathway signaling and induced apoptosis in LNCaP prostate cancer cells. Cholesterol replenishment activated Akt signaling and avoided apoptosis. Unfortunately, they used simvastatin concentrations of 20 μM, a level impossible to achieve in patients. This seminal finding clearly shows the mechanism by which cholesterol has an anti-tumorigenic behavior, but due to the excessively high simvastatin concentration it does not allow possible therapeutic conclusions. Simvastatin, as important as it is, is also a poison at very high concentrations, following Paracelsus principle: *sola dosis facit venenum.* Therefore, an unanswered question remains: can statins have the same effects at the concentrations they reach in human beings?

► Wong et al. showed that clinically achievable concentrations of statins had the ability to induce apoptosis in malignant cells through down-regulation of the anti-apoptotic protein bcl-2 [8,9]. The pro-apoptotic effect of statins was confirmed in many different tumor cell lines, including juvenile monomyelocytic leukemia [10], acute myeloid leukemia [11], myeloma [12], mesothelioma, pancreatic, colon [13], and prostate cancers [14], among others. However, when these experiments were analyzed in depth, the concentrations of lovastatin used were at the micromolar level while the achievable concentrations are in the nanomolar levels. This level is not feasible in the clinical setting. Thus, the tumor apoptotic effects of statins remain controversial [15].

► There are also experiments that take into account the achievable level of statins in blood. For example, Gordon et al. [16] administered oral simvastatin to LNCaP xenograft bearing castrated mice and the plasma level reached an average concentration of 3.29 nM without biochemical signs of toxicity. This level is found in humans taking 80 mg of the statin. The dose was effective to slowdown tumor growth and progression. Cholesterol *de novo* synthesis was also reduced. Can this be extrapolated to humans?

"Unfortunate" concentrations of statins raise doubts about the usefulness of some experiments and publications, but this does not preclude that in spite of these poorly planned experiments, the drug may have some anti-tumor effects.

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► Staying with statins let's go one step further. Many publications base the antitumoral action of statins on its ability to inhibit Ras farnesylation. Statins are inhibitors of HMG-CoA reductase, thus inhibitors of *de novo* cholesterol synthesis through the inhibition of mevalonate generation. Reducing cholesterol synthesis leads to a lower production of farnesylate which would decrease farnesylation of RAS and Rho GTPases and decrease its activation [17-20]. This mechanism is clearly anti-tumoral. However, it only works with very high concentrations of statins (50 μM). Surprisingly, Cho et al. [18] found that therapeutic levels of lovastatin, usually with a concentration range of 50 to 500 nanomoles (nM), not only did not decrease RAS activation, but increased it. This occurred as a consequence of phospholipase D activation. Therefore, therapeutic levels of lovastatin did not decrease RAS prenylation. In spite of this seminal finding, most of the publications repeat the mantra that statins decrease RAS signaling. This would only be true if highly toxic levels of statins were to be administered. This does not happen in patients treated with statins.

4. Metformin

Metformin is another paradigmatic drug to be repurposed almost for everything: aging, obesity, endometriosis, and fundamentally for cancer.

Since Evans et al. [19] in 2005, published their population-based statistical finding that metformin reduced the risk of cancer in diabetics, tons of papers have been published on this subject. Among half true and half erroneous concepts, some authors never really read the publication.

For example, an oncology book on repurposed drugs references the Evans manuscript saying that metformin has been used to treat cancer [20]. The Evans paper is a statistical study that shows a risk reduction of cancer in diabetics taking metformin for the treatment of diabetes, not for treating cancer. However, the authors say: "In 2005, MTF was used for breast cancer treatment."

These types of errors are not the focus of this manuscript. Let's analyze whether the research backing metformin as an "onco" drug is based on solid evidence.

What is the maximum non-toxic concentration of metformin?

Administering 500 mg of oral metformin to healthy volunteers the plasma concentration of metformin reached a maximum level of 1.42 μg/ml after two hours [21]. The usual daily dose does not exceed 3 g and the maximum approved total daily dose for diabetes mellitus is 2.5 g (35 mg/kg body weight) [22].

The concentration of metformin is high in the hepatic portal vein (approximately double than in cava vein), but after it emerges from the liver, the systemic plasma concentration is in the range of 10–40 μM in mice [23].

Reviewing the literature there is a wide range of metformin concentration in plasma which goes from 0.1 to 4 mg/l [24]. Therefore, we can assume 4 μg/ml to be the fair value. The molecular weight of metformin is 129.16 g/mol. We will use the maximal non-toxic concentration, 50 mg/l in humans (0.4 mM or 50μg/ml) as the upper limit of metformin that can be clinically reached with oral administration [25].

CONVERSION TABLE

4 μg/ml = 4 mg/liter = 0.004 g/l achievable plasma concentration with 500 mg metformin.

160 mg/liter was found in patients developing lactic acidosis with a mortality of 53% [26].

Molecular weight of metformin = 129.16 g/l

A solution with 1 Mol of metformin is a solution that contains 129.16 g/liter

1 mM of metformin is 129.16/1000 = 0.129 g/l = 129 mg/l = 129 μg/ml

Based on these data we analyzed some publications on metformin activity in cancer.

► Zhang et al. [27] studied the effect of 5 mM of metformin on CD133+ cells in colon cancer and arrived at the conclusion that "Inhibition of the proliferation of CD133+ cells may be a potential mechanism responsible for the association of metformin use with improved CRC outcomes in CRC patients with type 2 diabetes". 5 mM of metformin is the equivalent to 645 mg/l. This represents ~13-fold increase of what is a clinically achievable non-toxic concentration in plasma. To this we must add that the plasma concentration is always much higher than tumor concentration.

► Kim et al. [28] tested HeLa cells incubated with metformin. They found a decrease in proliferation starting at a concentration of 5 mM and from there on in a dose dependent manner. Again, the metformin concentrations were well beyond toxic levels.

► Jang et al. [29] used metformin for bladder cancer cells. A slight decrease of viability could be seen with metformin concentration of 3 mM. A clear drop in viability was only seen with concentrations between 9 and 12 mM. We are again in levels that are 20-fold higher than a tolerable dose.

► Griss et al. [30] showed that metformin decreased cancer cell proliferation *in vitro*, however they used concentrations between 2.5 and 10 mM.

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Table 1: Fenofibrate1's cytotoxicity on glioblastoma cells

Reference	Finding
Reiss et al., 2008 [47]	Fenofibrate sensitized glioblastoma cells to cisplatin.
Drukala et al. ,2010 [48]	Increased ROS potentiated fenofibrate's anti-glioblastoma effects.
Giordano et al., 2012 [49]	Fenofibrate induced apoptosis in glioblastoma cells.
Wilk et al., 2012 [50]	25 μM of fenofibrate induced glioblastoma cells G1 arrest with minimal apoptosis.. 50 μM achieved massive apoptosis. The mechanism was FOXO3 nuclear accumulation that induced BIM (an apoptotic protein) transcriptional activation.
Wilk et al., 2013 [51]	Anti-tumoral effects of fenofibrate on glioblastoma cells were found to be independent of PPARα agonism.
Binello et al., 2014 [52]	They found pro-apoptotic effects and CSCs migration inhibition with fenformin acting on glioblastoma cells.
Han et al., 2015 [53]	Fenofibrate inhibited NF-κB/RelA activation and by impeding its association with hypoxia inducible factor1 alpha (HIF1α), pyruvatekinase 2 is not expressed, thus favoring oxidative metabolism.
Grabacka et al. ,2016 [54]	Fenofibrate induced the production of ketone bodies in glioblastoma cells that cannot use them as an energy source, inducing growth arrest.
Kast et al., 2017 [55]	Fenofibrate decreased glioblastoma growth by decreasing/blocking glioblastoma induced granulocyte colony factor production.

The above mentioned publications were not selected, but randomly picked from Google Scholar under the search based on "metformin and cancer".

We are not saying here that metformin has no anti-cancer activity, as a matter of fact we believe exactly the opposite. The only objective is to show that many of the molecular mechanisms published as the fundamentals for metformin's anti-cancer effects, are based on concentrations that are absolutely impossible to achieve in patients. *In vitro* studies at poisonous levels, brings us back to Paracelsus concept and lack practical bedside application.

5. Capsaicin

It has been shown that capsaicin, present in hot peppers and chilli, has interesting anticancer effects. The most important seem to be promoting apoptosis in cancer but not in normal cells [31]. The anti-growth effects of capsaicin has been found in androgen- independent prostate cancer [32], squamous cell carcinoma KB cells [33], gastric [34], pancreatic [35], breast [36,37], colorectal [38,39], small cell lung cancer [40] cells among many others.

In spite of all this evidence favoring capsaicin, low concentrations have the ability to promote metastasis [41].

A research on capsaicin effects on renal cell carcinoma cells [42] showed that it had pro-apoptotic effects at an average concentration of 200 μM. One of the peer reviewers asked:

"Significant effect of Capsaicin was seen starting at the dose of 200 uM. What is the physiologically achievable concentration of capsaicin in Humans?"

Interestingly, the authors were unable to give a straight answer. They said: *"Lots of papers studied capsaicin at the concentration of 200 uM [1-3] (even 500uM [4]) in vitro cell models"*.

This means that they used 200 μM (equivalent to approximately 61 mg/ml. Capsaicin's molecular weight is 305 g/mol).) because other authors used the same concentration or even higher, but we still do not know if this concentration is clinically achievable. The only guide we found is that capsaicin in rats can reach 90 ng/ml in blood, 167 pg/mg in the lung and 3.4 pg/mg in the liver [43]. Suresh et al. [44] found an average concentration of 1.9 μg/ml after an hour of a high intake of capsaicin in rats. This level halved after another hour. In men, after a high intake of capsaicin containing food a level of 179 ng/ml was found [45].

Therefore, a concentration of 200 μM is far beyond the concentration found in rats and in humans. Prima facie, it seems very doubtful that 200 μM can be clinically achieved.

6. The Wrong Route

A different case is fenofibrate. This lipid lowering drug has clearly established antitumoral abilities [46], although not all the mechanisms are clearly known. Interestingly, there is a great deal of research going on regarding fenofibrate's activity on glioblastoma cells. We summarized them in Table 1.

From Table 1 the first conclusion would be that fenofibrate should be a first line treatment for glioblastoma. Furthermore, PPARα overexpression was found to be associated with a better prognosis in wild type glioblastoma [56] and precisely fenofibrate is a PPARα agonist.

However, there is a problem that some authors seem to forget: "fenofibrate does not cross the blood brain barrier and is quickly processed by blood and tissue esterases to form the PPARα agonist fenofibric acid, which is practically ineffective in triggering cancer cell death" [57,58]. Therefore until a practical approach to deliver fenofibrate into the brain tumor is found, all the research at the cellu-

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lar level will remain in the laboratory. This we have called the administration route bias.

However, there are some authors that even recommended using oral fenofibrate (100 mg twice a day) in the clinical setting for glioblastoma as part of a multidrug scheme [59]. Unfortunately, they offer no proof of any benefit.

There is enough evidence to include fenofibrate in a clinical trial as a complementary drug for diverse cancers, except glioblastoma. As soon as the delivery system beyond the blood-brain-tumor barrier for fenofibrate becomes reality, this will change. At the present moment recommending the unmodified fenofibrate for glioblastoma in the clinical setting is at least a poor idea. If there are authors that pretend to use unmodified fenofibrate for glioblastoma, we wander if they pretend to drill a hole in the skull to smear 100 mg twice a day on the tumor. Otherwise, they will only lower triglycerides and we do not think that will change the course of events.

7. Silymarin

Is a compound obtained from milk thisthle (Sibylium marianum) that has been used for more than two thousand years for the treatment of diverse ailments. Nowadays, it is prescribed for the therapy of liver toxicity produced by ingestion of Amanita phalloides mushroom, and other chemicals and for non-alcoholic liver esteatosis. In the last twenty-five years silymarin and its main active principle, silybin, has been under scrutiny for the treatment of cancer [60].

SIL is not soluble in water and oral administration shows poor absorption in the alimentary tract (approximately 1 % in rats [61], however, other authors mention a higher absorption around 30%); it is mainly excreted in the bile. Toxicity is almost absent [62] and therefore high oral doses can be administered with negligible side effects.

In spite of this low absorption, according to Janiak et al. a plasma level of 500 mg/L (500 μg/ml) is achievable 90 minutes after oral administration of 200 mg/kg of silymarin in mice [63]. The elimination half-life is 6 hours.

240 mg of silybin were orally administered to six healthy volunteers and the following results were obtained: **maximum plasma concentration 0.34 ± 0.16 μg/ml** and time to maximum plasma concentration 1.32 ± 0.45 h. Absorption half life 0.17 ± 0.09 h, elimination half life 6.32 ± 3.94 [64].

Beckmann-Knopp et al. [65] found: *"Mean maximum plasma concentration after an oral dose of 700 mg silymarin, containing 254 mg of silibinin, is* ***317 ng/ml or 0.6 mM.*** *Accumulation in plasma during three daily medications is negligible. Plasma protein binding is reported to reach about 90–95%".*

Gatti et al. [66] found that free unconjugated silybin reached a maximum concentration of 141 ng/ml after 2.4 hours of feeding volunteers with 80 mg of a lipophilic silybin-phospatidylcholine complex (silipide). The level of conjugated silybin peaked after 3.8 hours reaching 255 ng/ml.

Another study on 6 healthy volunteers receiving 560 mg of silymarin attained concentrations ranging between 0.18 to 0.64 μg/ml [67].

In dogs [68], the silybin-phosphatidylcholine complex (SPC) showed increased concentration when compared with silymarin extract, however, the results show a low level in general:

SPC: 1,310 ± 880 ng/ml; silymarin: 383 ± 472 ng/ml.

Morazzoni et al. [69] found higher peak levels of silybin in the form of silipide when administered to rats: *"After oral silipide, silybin reached peak plasma levels within 2 h, with a* C_{max} *of 9.0±3.0 μg/ml for unconjugated drug and 93.4±16.7 μg/ml for total (free + unconjugated drug)".*

Concentration in humans (with a low dose) is far lower than what was found in rodents (with a high dose). The important issue to raise is that most of the experiments at cellular level that can be found in the literature use a concentration around 100 μg/ml. Even in the publication of Morazzoni the level of 100 μg/ml was not achieved and in any case it is a peak level that cannot be sustained. Therefore, is the experimental level achievable at the bedside?

We think that there is no evidence that it can be.

Oral administration of SIL in humans achieves nanogram levels but not micrograms. Furthermore, we should not extrapolate Morazzoni et al. findings in rats to humans.

Therefore, the evidence based on these high concentration experiments should be cautiously viewed.

8. Discussion

Five drugs, namely statins, metformin, capsaicin, fenofibrate,and silybin are considered in many papers as candidates to be repurposed for cancer treatment. The first three were used against cancer in *in vitro* and *in vivo* experiments. Many of the published articles in medical literature show that these drugs were essayed in concentrations that in most cases are impossible to achieve in patients.

The fourth drug, fenofibrate, was proposed at an oral dose of 100 mg twice a day for the treatment of glioblastoma when this drug is unable to cross the blood-brain barrier. Regarding silymarin and silybin the clinically achievable concentration may be enough for its antimigratory effects but are insufficient to induce apoptosis.

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Drug repurposing is a growing and useful research activity, however we cannot avoid asking how useful many of these publications are when they use these drugs under conditions that are impossible to attain in patients.

It is quite possible that silibyn serum concentration will be improved with the new pharmaceutical forms under research. Being non-toxic even at high doses, gives a good possibility to improve its anti-cancer scope. On the other hand, metformin represents an absolute barrier due to its toxicity. Achieving a higher plasma concentration will only increase the chances of lactic acidosis.

9. Conclusions

Some ongoing research on drug repurposing seems to belong more to the realm of Cesare Borgia than to reasonable and scientific molecular pharmacology. According to some not very reliable twisted stories, Borgia was a notorious master poisoner in sixteenth century Rome. If we poison the cell, it will surely die. Paracelsus cautioned us that we must use drugs within their therapeutic range. This seems to be ancient history for many twenty first-century researchers.

Some authors take for granted that what happens in the Petri dish, no matter the concentration of the drug, will also happen in the patients. The problem is that they forget that a drug to be effective has to reach its target.

Dose bias and administration route bias seem to represent a substantial part of present day cancer research.

Finally, we should ask how reliable peer review is, when all these supposedly reviewed but heavily biased articles pullulate in prestigious journals and in the web.

Paraphrasing Paracelsus: *Venenose portione vir necat, autem non sanat*.

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ARTICLE

In vitro Activity of Novel Cannabinoids Derived from Tetrahydrocannabinolic Acid on Various Human Tumor Cell Lines

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ABSTRACT

The *in vitro* study of tetracannabinolic acid (THCA) derivatives ALAM027 and ALAM108 was carried out on the following human tumor cells: T47D (breast, ductal carcinoma), PC-3 (prostate, adenocarcinoma), HT-29 (colorectal carcinoma), Caco-2 (colon, adenocarcinoma), A549 (lung, carcinoma), U87MG (human glioblastoma) and U266B1 (multiple myeloma).

The *in vitro* effects of THCA derivatives ALAM027 and ALAM108 on cell growth inhibition and IC50 values were measured using the CellTiter Glo assay.

The ALAM027 compound showed good growth inhibition in all cell lines tested with the exception of U87MG cells. The ALAM108 compound also suppressed the growth of U87 MG cells but had little effect on T47D tumor cells.

In vitro studies of THCA derivatives ALAM027 and ALAM108 showed antitumor activity in all cell lines tested. The difference in the activity of these compounds in relation to the T47D and U87MG tumor cells may be indicative of different functional mechanisms.

1. Introduction

Tetrahydrocannabinolic acid (THCA) is the main component of *Cannabis sativa*. However, in contrast to its derivative THC, the biological properties of THCA have been studied to a much lesser extent, particularly because it is difficult to isolate and because of its high sensitivity to heat and UV radiation. A convenient and inexpensive method has recently been described to isolate this acid using ion-exchange resins, opening up the way to industrial scale production and making THCA a suitable starting product for drug synthesis [1,2]. This advance has recently facilitated the synthesis of two THCA derivatives, ALAM027 and ALAM108, which exhibit good anti-tumor activity in PANC-1 and AsPC-1 cell lines [3].

Since natural cannabinoids such as THC and CBD are known to have broad-spectrum anti-tumor activity against many types of tumors [4-6], it is of interest to investigate a potential effect of ALAM027 and ALAM108 on various types of cancer cells. According to World Health Organization data the most widespread types of cancers are breast, lung, colon, intestine, pancreatic, prostate tumors and blood diseases such as multiple myeloma. Brain tumors such as gliomas are also potentially interesting, particularly because of their aggressive and highly invasive properties [7]. To facilitate comparisons between previously reported activities of natural cannabinoids and the

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ALAM027 and ALAM108 compounds, the current study examines the effects of these compounds on the following human cancer cell lines: T47D (breast, ductal carcinoma), PC-3 (prostate, adenocarcinoma), HT-29 (colorectal carcinoma), Caco-2 (colon, adenocarcinoma), A549 (lung, carcinoma), U87MG (human glioblastoma) and U266B1(-multiple myeloma).

All these tumor cell lines express significant levels of CB1 and CB2 receptors in their cell membrane and this amount increases with cell proliferation. Thus, several articles have been devoted to studying the effects of THC and CBD on tumor cell line T47D which is often used in breast cancer research [8-11].

The PC-3 cell line is also often used in prostate cancer research. Studies examining the effects of cannabinoids on PC-3 cells have predominantly focused on elucidating tumor growth suppression mechanisms [12,13].

The HT-29 cell line is a colorectal tumor line which is often used as an epithelial cell model to study new drug candidates because of its ability to differentiate. Cannabinoids have a significant effect on HT-29 cells as they induce cell death through apoptosis and inhibit proliferation [14]. The role of cannabinoid receptors in these processes has been studied by examining effects of agonists such as THC and CBD on cancer cells in the presence and absence of CB1 and CB2 antagonists [15].

Similarly to HT-29 cells, natural cannabinoids also suppress colorectal adenocarcinoma Caco-2 cell growth by inducing apoptosis and inhibiting cell proliferation which is mediated through CB1 receptor binding [16,17].

A549 is one of the most widely studied lung tumor cell line, which is often used as a testing ground for new drugs, such as natural and synthetic cannabinoids because the main determining factor of the anticancer effect of these cannabinoids on A549 cells is their ability to block the CB1 receptor, which is overexpressed in non-small cell lung tumors [18-20].

Brain glioblastoma occupies an important place among studies of the anticancer activity of cannabinoids. A significant number of research studies have focused on the effects of THC and CBD ligands on U87 MG, in particular because of their rather substantial *in vitro* and *in vivo* activities [21-23]. It is interesting to note that, in contrast to SF126 glioblastoma cells, in the case of U87MG cells THC does not exhibit a pleiotropic effect at 1 μM concentration and below [21]. Multiple myeloma is one of the most serious hematological diseases and is characterized by drug resistance. Cannabinoids are among the most promising candidates for the treatment of this disease. Recent research [24] indicates that the IC50 values for CBD and THC in U266 cells are 19.8 μM and 39.5 μM respectively, and their combined use leads to the synergistic increase of cytotoxic effects when compared to their individual activities.

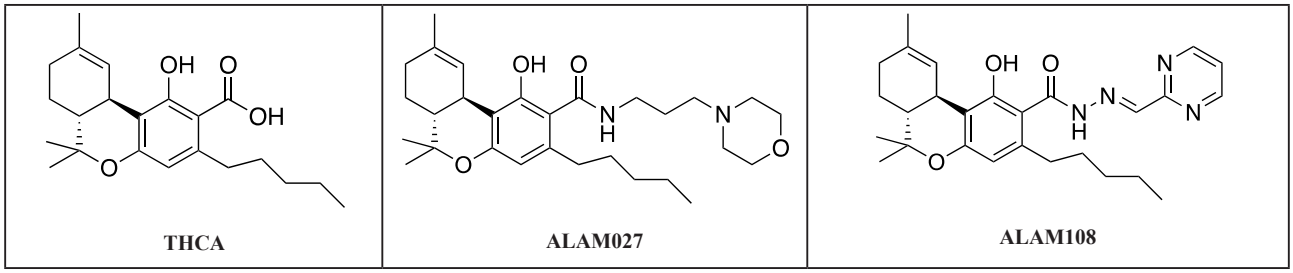
The present activity study of the ALAM027 and ALAM108 compounds in these selected tumor cell lines will not only allow to assess their anticancer activity but also, to a certain extent, could be used to understand their putative functional mechanisms.

2. Materials and Methods

Synthesis data and spectral characteristics of THCA derivatives ALAM027 and ALAM108 have been described previously [8]. The *in vitro* study was performed on T47D, PC-3, HT-29, Caco-2, A549, U87MG and U266B1 cells obtained from the Chempartner (China) collection using the CellTiter Glo Viability Assay. Cells were seeded in 96-well plates in a volume of 100 μl per well, according to the planned plate layout and a predefined seeding density. Plates were incubated in a CO_2 incubator overnight. The compound stock solution was diluted with DMSO to a 200 × final concentration, and serial 3-fold dilutions prepared from a 2-mM solution (final concentration range: 0.5-10000 nM for 10 doses). An internal staurosporine control was included on each plate. A volume of 0.5 μl of diluted compound was added to appropriate wells according to the plate layout. The plates were incubated at 5% CO_2, 37°C for 72 hours. After this incubation, CellTiter-Glo reagents were prepared and added, and the plates read in an Envision plate reader. Inhibition and IC50 for each of the compounds were calculated with the XLFit curve fitting software (n=2, Z Factor, SW).

3. Results and Discussions

The structures of THCA and its derivatives ALAM 027



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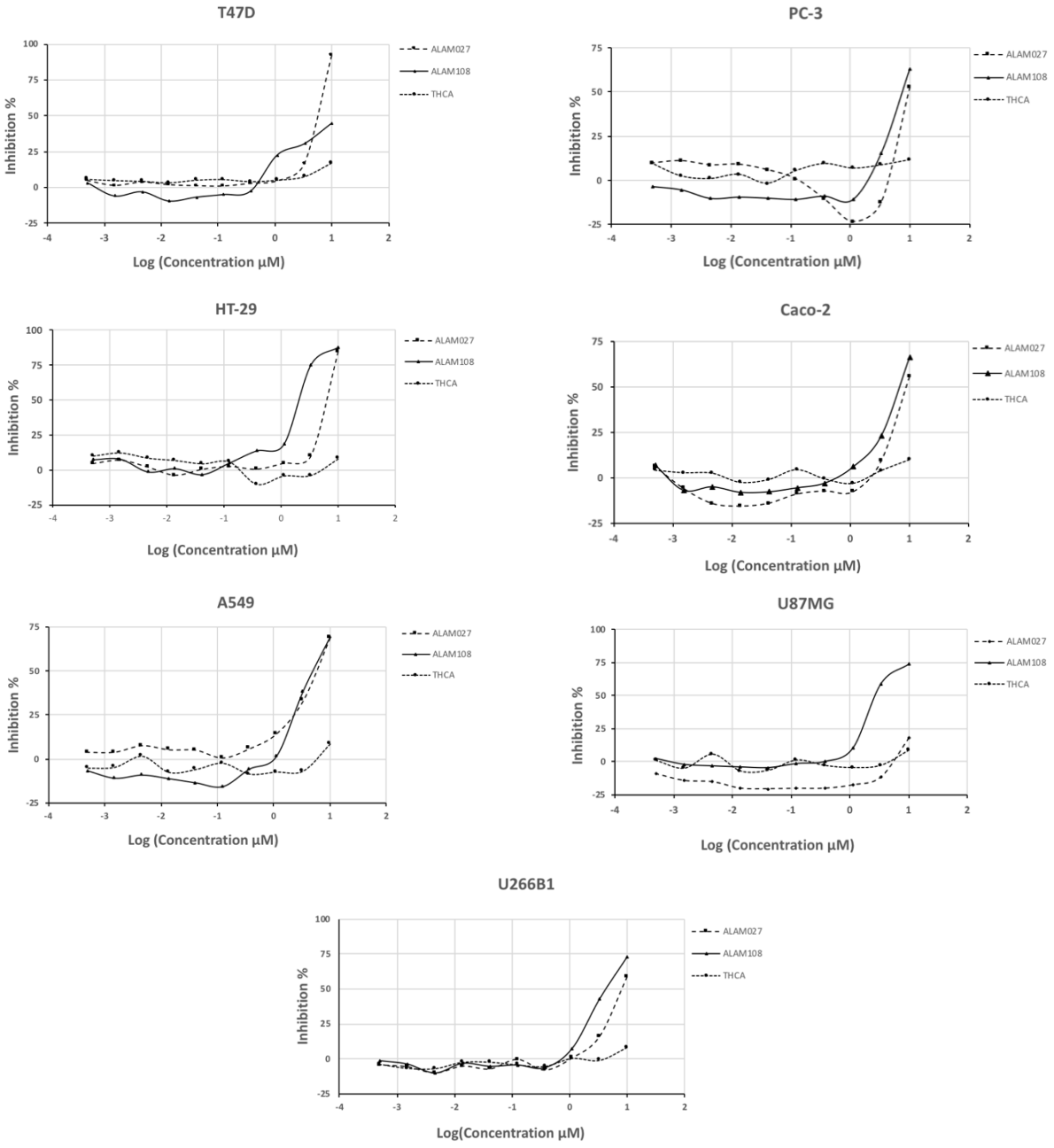

Figure 1. Growth inhibition curves of T47D, PC-3, HT-29, Caco-2, A549, U87MG and U266B1 cells at a wide range of THCA, ALAM027, and ALAM108 concentrations.

and ALAM108 are shown below.

The growth inhibition curves of T47D, PC-3, HT-29, Caco-2, A549, U47MG and U266B1 tumor cells as a function of THCA, ALAM027 and ALAM108 concentrations are shown in Figure 1.

Compared to its derivatives the level of THCA growth inhibition, was generally low for all cell lines examined but THCA did tend to inhibit T47D, A549 and U87MG cell proliferation to a greater extent than the other lines (Table 1).

For the T47D cell line, the ALAM027 compound shows good growth inhibition with an IC50 value comparable to THC (6.7 µM) and CBD (5 µM) [8]. The ALAM108 compound is less active, though its ability to inhibit cell proliferation significantly exceeds that of THCA.

The inhibition values of both cannabinoids on PC-3 prostate tumor cells are very similar (Table 1) but the ALAM027 compound yields a pleiotropic effect at 1 µM concentration following the growth inhibition.

The comparison of both cannabinoid activities in

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HT-29 cells shows that these cells are more sensitive to ALAM108 than to ALAM027 while IC50 values of both compounds are much higher than those of CBD (23-30 μM) and THC (30 μM) [14]. However, in the case of Caco-2 cells the IC50 values differ slightly and are comparable to those obtained in PC-3 cells.

The effect of ALAM027 and ALAM108 on A549 cells is practically the same both in terms of the degree of inhibition and IC50. When compared to THC (27.2 μM) and CBD (37.1μM) [19] this advantage becomes clearly evident.

Regarding the U87MG cell line, compound ALAM027 shows a low-level activity comparable to THCA. Cannabinoid ALAM108 effectively inhibits cell survival with an IC50 of 3.37 μM that is on par with the activity of THC (IC50 1.2-14 μM) and CBD (IC50 1.5-9.7 μM) in this cell line [21]. One of the possible reasons may be the ability of ALAM108 to pass through the blood-brain barrier due to its greater hydrophobicity (LogP 5.81) compared to ALAM027 (LogP 4.38). Perhaps this assumption is very relative, but currently available cannabinoid anticancer activity data on U87MG cells only relates to THC, CBD and some synthetic cannabinoids like WIN55,212-2 [19-23].

The comparison of both cannabinoid activity against U266B1 cells shows the advantage of ALAM108 as for other cell lines.

Table 1. IC50 and inhibition values (10 μM) of THCA and its derivatives on T47D, A549, PC-3, HT-29, Caco-2, U87 MG, and U266B1 tumor cells.

Cancer cell lines	THCA		ALAM027		ALAM108		Parameters of screening assay*	
	Inhibition%	IC_{50} μM	Inhibition%	IC_{50} μM	Inhibition%	IC_{50} μM	Z Factor	SW
T47D	18.20	>10	97.90	5.52	47.20	>10	0.86	20.42
U87MG	10.52	>10	19.84	>10	73.80	3.37	0.93	45.37
A549	9.30	>10	77.08	5.59	70.01	5.53	0.83	17.22
PC-3	15.43	>10	61.13	9.94	63.61	7.45	0.81	12.68
HT-29	16.77	>10	86.21	6.27	88.13	1.99	0.85	18.99
Caco-2	12.99	>10	60.81	8.87	67.16	6.56	0.80	13.02
U266B1	8.68	>10	58.33	8.20	73.05	4.52	0.88	25.03

* [25]

4. Conclusions

Our current *in vitro* study of THCA derivatives ALAM027 and ALAM108 showed their antitumor activity in all the tumor cell types examined. The difference in the activity of these compounds in relation to the T47D and U87MG tumor cells may be indicative of different functional mechanisms.

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Author Disclosure Statement

The author has no conflicts of interest, and no competing financial interests exist.

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ARTICLE

Functional Outcomes of Limb Salvage Surgery in Patients with Giant Cell Tumor of Bone of the Lower Extremities: A Retrospective Study

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ABSTRACT

Giant cell tumor of the bone (GCTB) is a benign, locally aggressive neoplasm that is relatively rare, with a propensity to result in progressive bone destruction, and is associated with a high risk of recurrence. There is no widely held consensus regarding its ideal treatment. Worldwide, there are varying techniques ranging from intralesional curettage to resection of the lesion, supplemented with combinations of numerous adjuncts and fillers, depending on the resected amount and integrity of bone, as well as the preference of the surgeon.

This was a cross-sectional study that included 20 patients who underwent limb salvage surgery for giant cell tumor of the bone of the lower extremities from January 2009 to February 2020 at two tertiary hospitals.

The mean follow-up period was 37.3 months (SD=2.84). The extended curettage (EC) group had a mean Musculoskeletal Tumor Rating Scale (MSTS) score of 28.18 (SD=7.51) which is considered as an excellent outcome, while the resection (RS) group had an mean MSTS score of 19.67 (SD=11.02), which is considered as a good outcome. EC resulted to a total of eight complications (47%), while RS had one complication (33%). Prevalence of recurrence was noted to be 11.75% among those who underwent EC, while no recurrence was noted among those in the RS group. Use of bone cement as a filler was noted to have less recurrence as compared with the use of bone grafts, however were both were noted to result in excellent functional outcomes.

Despite the prevalence of complications and recurrence of GCTB of the salvaged extremity in those who underwent EC, there is still report of excellent functionality. It is hence important to disclose all these possible outcomes and to stress the importance of compliance to follow-up for monitoring of these events.

1. Introduction

Giant cell tumor of the bone (GCTB) is a benign but locally aggressive neoplasm comprising approximately 5% of all primary bone tumors [1-6,9,15,16,18,20]. The natural history of GCTB is progressive bone destruction leading to joint deformity and disability, and despite it rarely causing death, it displays a tendency for local recurrence occurring within two years of the index surgery, and pulmonary metastasis (1.8-9.1% of cases) has also been described [1,2,5,6,14,15,21]. There are documented cases of malignant transformation in less than 1% of GCTs and are hence considered rare [1,5,6].

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There is no widely held consensus regarding the ideal treatment method selection of GCTBs. Options of chemotherapy and radiotherapy are reserved for select cases however evidence shows no clear benefit for its use in these cases [2,14]. Treatment has become mainly surgical and is the universal standard of care due to the association of GTCB with substantial disturbance of local bony architecture especially in periarticular locations [2,4-6,14,15]. In our institution, as well as worldwide, there are advocates of varying surgical techniques ranging from intralesional curettage to wide excision of the lesion, supplemented with cement augmentation and fixation with implants, depending on the amount of bone resected, integrity of the articular surface, and the preference of the surgeon [2].

Regardless of technique, the goal of limb-salvage are eradication of the tumor, preservation of limb function, and prevention of local recurrence and distant metastasis [2,5,14], as some studies show a correlation of the rate of local recurrence varying with the extent of GCT removal [2,3,6,22]. Patients with wide resection of the tumor are noted to have a local recurrence rate approaching 0% [6], but with these leading to higher rates of surgical complications and may lead to functional impairment, necessitating reconstruction [2,3,18]. On the other hand, intralesional curettage despite resulting in less morbidity and functional impairment, regardless of how thoroughly performed, leaves microscopic disease and hence has a reported recurrence rate as high as 60-65% [2,3,14,18,20]. This has led surgeons to enhance surgical procedures with the use of adjuvants such as liquid nitrogen, cement, phenol, hydrogen peroxide, which in some studies, have shown to be useful in decreasing recurrence rates [2,4].

In the Philippines, options for limb-salvage surgery for malignant and aggressive extremity neoplasms after tumor excision are limited as they would often entail the use of bone grafts, not only due to the sheer size of the defects left in the aftermath of the procedure, but also due to the unavailability and unaffordability of tumor prosthesis and implants [4], often resulting to amputation of the involved extremity. In this developing country, there are only a few centers with an active bone bank, and our institution is honored to house one of them, for which a stock of allografts are available for use intraoperatively.

To date, possibly owing to the relative rarity of the disease, there has been no other published work in regards to the functional outcomes of patients in the Philippines diagnosed specifically with GCTBs of the lower extremity who underwent limb-salvage surgery, as well as the complication and recurrence rates following the aforementioned procedure. The researchers would like to provide evidence for this gap in knowledge, as well as to determine the outcomes of the usage of allografts in this population who have access and are able to afford this modality.

2. Materials and Methods

Study Design and Procedure

This was a cross-sectional study investigating patients who underwent limb-salvage surgery of the lower extremity for GCTB from January 2009 to February 2020 at two tertiary hospitals. Limb salvage surgery in this study is defined as resection (RS) or extended curettage (EC) of the GCTB lesion of a Campanacci grading of II or III, with or without the use of local adjuvants, with the goal of eradication of the tumor, preservation of the limb and its function, and prevention of recurrence.

Due to the relative rarity of the disease, convenience sampling of the cases of interest was done. A thorough review of the patient's electronic hospital records (Enterprise Portal v1.6.1 rev.219), including the operative record for details regarding the surgery was done. All information was collated using a data collection tool.

Functional outcomes, on the other hand, were derived from the surgeon's clinic charts of the patient's subsequent follow-up visits postoperatively. These were rated using the Musculoskeletal Tumor Rating Scale (MSTS), a validated questionnaire developed initially by Enneking et al in 1993 and has been in use for over 20 years as a widely recognized tool to evaluate function. This system measures outcomes in seven categories, including motion, pain, stability, deformity, strength, activity, and emotional acceptance, specified to the anatomic location of interest (ie, hip, knee, or ankle) [8]. Each parameter is scored 0-5 and combined for a possible total score of 35. A score of 23 or greater is considered an excellent result; a score of 15-22 is considered a good result; a score of 8-14 is considered a fair result; and lastly, a score of less than 8 is considered a poor result, in terms of functionality [9].

Likewise, the occurrence of any complication or recurrence was noted using these records. The development of progressive lucency at the cement-bone interface in radiographs, or the presence of osteolysis and presence of a soft tissue mass in CT or MR imaging following surgery suggests recurrence of GCTB [6]. A complication on the other hand is defined as any event for which the patient required a specific intervention such as wound complications, infection, implant failure/loosening, fracture, and stiffness of the joint [4].

Approval was first obtained from the Institutional Review Board and Ethics Committee of our institution prior to the commencement of this study.

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Eligibility Criteria

Inclusion criteria for the patients to be recruited were as follows:

(1) More than 18 years old during time of the diagnosis of GCTB and of limb-salvage surgery;

(2) Diagnosed with GCTB of the lower extremity via imaging (radiographs, CT scan, MRI);

(3) Classified with GCTB Campanacci grade II or III who underwent limb-salvage surgery, at either St. Luke's Medical Center-Quezon City or Bonifacio Global City;

(4) Has a histopathology result confirming the diagnosis of GCTB;

(5) With active follow-up of up to at least 6 months post-operatively;

Exclusion criteria are as follows:

(1) Those with open wounds, skin lesions directly overlying the surgical area, and/or active infections (either local or systemic);

(2) With pre-surgical conditions or comorbidities other than GCTB rendering the patient unable to ambulate or do range of motion of the lower extremities;

(3) With incomplete medical data from either hospital or clinic records;

Study Procedures

Due to the relative rarity of the disease, convenience sampling of the cases of interest was done. After these select cases were listed, identification of the attending surgeons was done, each of which were individually contacted to inform them of the eligibility of their patient/s.

After their confirmation, a thorough review of the patient's database and electronic hospital records (Enterprise Portal v1.6.1 rev.219) during his/her admission, including the operative record for details surrounding the surgery will also be done. Follow-up data, on the other hand, were obtained from the surgeon's clinic charts of the patient's subsequent visits postoperatively to determine information on his/her functional outcomes, which were completed by the investigator using the MSTS questionnaire. All information obtained will be collated using a data collection tool.

Objectives

The general objective of this study aimed to determine the outcomes of limb-salvage surgery in patients diagnosed with GCTB of the lower extremities. The specific objectives were to obtain the following:

Demographic and surgical profile of the selected participants.

Functional outcomes among participants using the Musculoskeletal Tumor Rating Scale (MSTS) score in terms of their motion, pain, stability, deformity, strength, activity, and emotional acceptance. This will be determined according to surgical techniques as well as according to the use of fillers. Each parameter is scored 0-5 and combined for a possible total score of 35. A score of 23 or greater is considered an excellent result; a score of 15-22 is considered a good result; a score of 8-14 is considered a fair result; and lastly, a score of less than 8 is considered a poor result, in terms of functionality [9].

Recurrence and complications among the participants. This will also be determined according to surgical techniques and use of fillers. The development of progressive lucency at the cement-bone interface in radiographs, or the presence of osteolysis and presence of a soft tissue mass in CT or MR imaging following surgery suggests recurrence of GCTB [6]. A complication on the other hand is defined as any event for which the patient required a specific intervention such as wound complications, infection, implant failure/loosening, fracture, and stiffness of the joint [4].

Sample size estimation

Sample size was calculated based on the estimation of the population proportion for functional score (MSTS). Assuming that the proportion of post-limb salvage surgery in patients with primary bone tumors with good to excellent results is 90% [9], with a maximum allowable error of 7.5%, and a reliability of 80%, the sample size required is 27.

Statistical Analysis

Statistical analyses were performed using STATA Statistical Software, Version 13, College Station, TX; StataCorp LP. Descriptive statistics involved mean, standard deviation, frequency, percentage, median, and interquartile range. Descriptive statistics on the functional outcomes, and complication occurrence and recurrence was estimated using chi-square test exact binomial with a 95% confidence interval (95% CI). All valid data were included in the analysis. Missing variables was neither imputed nor estimated.

3. Results

We analyzed a total of 20 patients diagnosed radiographically and histologically with giant cell tumor of the bone of the lower extremities that underwent limb salvage surgery in our institution from January 2009 to February 2020. Table 1 illustrates the demographic and surgical profiles of the respondents. It can be noted that the mean age of the respondents was 31.70 years (SD=9.44). Ma-

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jority of the respondents were males (55%), had femoral distal third involvement (40%), and had a Campanacci score of III (55%). There is a mean tumor size of 4.12 cm (SD=2.20) anteroposteriorly, 4.92 cm (SD=2.78) craniocaudally, and 4.92 cm (SD=2.73) transversely. Majority of the respondents underwent EC (85%) along with electrocautery with hydrogen peroxide (20%). The most commonly used filler was bone cement (35.29%). The mean operative time was 4.68 hours (SD=1.87), with a mean intraoperative blood loss of 549.00 milliliters (SD=764.97). The mean duration of hospital stay was 5.25 days (SD=2.84). There was note of shorter duration of operative time (EC 4.28 hrs vs RS 6.94 hrs), less intraoperative blood loss (EC 351.76 mL vs RS 1,666.67 mL), and shorter hospital stay (EC 4.53 days vs RS 9.33 days), in favor of the EC group. The duration of follow-up visit ranged from 6 months to 8 years, with a mean of 37.30 months (SD=30.64). It was also observed that among all the respondents, only 25% (5/20) of the respondents had used denosumab.

Table 2 illustrates the descriptive statistics of the MSTS score according to surgical techniques. It is noted that

Table 1. Demographic and Surgical Profiles of the Respondents (N = 20)

Characteristics	Frequency (f)	Percentage (%)	Mean (SD)
Age (Year)			31.70 (9.44)
Sex			
Male	11	55.00%	
Female	9	45.00%	
Bone Involvement			
Tibia – Proximal Third	7	35.00%	
Tibia – Distal Third	1	5.00%	
Femur – Proximal Third	3	15.00%	
Femur – Distal Third	8	40.00%	
Foot	1	5.00%	
Tumor Size			
Anteroposterior			4.12 (2.20)
Craniocaudal			4.92 (2.78)
Transverse			4.92 (2.73)
Campanacci Grade			
II	9	45.00%	
III	11	55.00%	
Surgical Technique			
Resection	3	15.00%	
Extended Curettage			
Electrocautery	2	10.00%	
Electrocautery with Phenol	2	10.00%	
Electrocautery with Burr	2	10.00%	
Electrocautery with Burr and Phenol	2	10.00%	
Electrocautery with Hydrogen Peroxide	4	20.00%	
Electrocautery with Burr and Hydrogen Peroxide	3	15.00%	
Electrocautery with Hydrogen Peroxide and Ethanol	2	10.00%	
Use of Fillers			
Bone Cement	6	35.29%	
Femoral Head Allograft alone	1	5.88%	
Hydroxyapatite Crystals alone	3	17.65%	
Femoral Head Allograft with Autograft	3	17.65%	
Femoral Head Allograft with Bone Cement	3	17.65%	
Femoral Head Allograft with Bone Cement and DBM	1	5.88%	
Intraoperative Blood Loss (Milliliters)			549.00 (764.97)
Resection			1,666.67(1,154.70)
Extended Curettage			351.76 (502.67)
Duration of Operation Time (Hours)			4.68 (1.87)
Resection			6.94 (1.42)
Extended Curettage			4.28 (1.67)
Duration of Hospital Stay (Days)			5.25 (2.84)
Resection			9.33 (5.13)
Extended Curettage			4.53 (1.62)
Duration of Follow-up (Months)			37.30 (30.64)

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among the different variants of EC, the use of electrocautery with phenol produced the highest MSTS score of 34 (SD=1.41). Overall, the approaches of EC had a mean score of 28.18 (SD=7.51) which is considered generally as an excellent outcome, while RS had a mean score of 19.67 (SD=11.02), which is generally considered as a good outcome.

The descriptive statistics of the MSTS score according to the use of fillers are presented in Table 3. It is noted that the use of femoral head allografts in general resulted in excellent outcomes (mean 24.37, SD=9.10), similar to the use of bone cement (mean 31.33, SD=5.12), and hydroxyapatite crystals (mean 31.00, SD=2.00).

Table 4 shows the descriptive statistics for the prevalence of complications and recurrence according to surgical technique. As demonstrated, among 17 cases of EC, there was a total of 8 complications (47%), including contractures (2), implant irritation (2), osteoarthritic changes (2), iatrogenic fracture (1), and arthrofibrosis (1). Among the three cases of RS, on the other hand, only 1 complication of postoperative infection (33%) was noted. Recurrence overall was 2 out 20 cases (10%) but segregated to technique, it was not noted in the resection group, while both cases were from the EC group (11.76% [2/17]).

The descriptive statistics of the prevalence of complications and recurrence according to the use of fillers are presented in Table 5. Five patients among those who utilized femoral head grafts (62.5%) as fillers were noted to develop complications (3/5 developed more than one complication). Likewise, both recurrences noted in this study both had use of femoral head allografts (25%).

4. Discussion

GCTB accounts for only 5% of all primary bone tumors [1-6,9,15,16,18,20] and are known to be locally aggressive benign tumors, and a propensity to be highly recurrent but with a rare metastatic potential [1,5,6,14,15]. Most of these lesions develop in long bones (75%-90%), with majority of cases (50-65%) occurring adjacent to the knee [2,5,6,9,16,18]. This was similar to our study, which showed the most commonly affected sites being the distal femur (40%) and proximal tibia (35%). Due to its common affliction in these areas, the natural history of GCTB leads to morbidity resulting from the substantial disturbance of the local bony architecture of these periarticular locations [2,3,9,21]. Although some studies show an equal sex distribution, most show a slight predilection among females [2,3,5,6,15,22]. This was in contrast to our study, as it was noted that the majority of our population (55%) comprised of males. Multiple studies have likewise shown that GCTB may occur in any age group but is observed to peak during the 3rd decade, with 80% of cases occurring between 20-50 years of age [3,5,6,15,20,22]. This is in concordance with our study, showing a mean age of 31.70 years.

The high suspicion for a diagnosis of GCTB begins with a typical radiographic presentation of a lytic bony

Table 2. Descriptive Statistics of Musculoskeletal Tumor Rating Scale (MSTS) according to Surgical Technique among the Respondents (N = 20)

Musculoskeletal Tumor Rating Scale	Surgical Technique								
		Extended Curettage (N=17)							
	Resection (N=3)	Overall	Electrocautery (N=2)	Electrocautery with Phenol (N=2)	Electrocautery with Burr (N=2)	Electrocautery with Burr and Phenol (N=2)	Electrocautery with Hydrogen Peroxide (N=4)	Electrocautery with Burr and Hydrogen Peroxide (N=3)	Electrocautery with Hydrogen Peroxide and Ethanol (N=2)
Overall Score	19.67 (11.02)	28.18 (7.51)	31.00 (0.00)	34.00 (1.41)	25.00 (11.31)	27.00 (8.49)	29.00 (5.66)	27.33 (11.59)	23.50 (13.44)

Values are presented as mean (standard deviation).

Table 3. Descriptive Statistics of Musculoskeletal Tumor Rating Scale (MSTS) according to Use of Fillers among the Respondents (N = 17)

Musculoskeletal Tumor Rating Scale	Use of Fillers (N=17)						
	Bone cement (N=6)	Hydroxyapatite crystals(N=3)	Overall use of femoral head allografts (N=8)	Femoral head allograft alone (N=1)	Femoral head allograft with bone cement (N=3)	Femoral head allograft with autograft (N=3)	Femoral head allograft with bone cement and demineralized bone matrix (N=1)
Overall Score	31.33 (5.12)	31.00 (2.00)	24.37 (9.10)	35.00 (0.00)	17.00 (3.60)	26.67 (10.97)	29.00 (0.00)

Values are presented as mean (standard deviation).

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Table 4. Descriptive Statistics of the Prevalence of Complications and Recurrence among the Respondents according to Surgical Technique (N = 20)

Complications (N=8)	Surgical Technique						
		Extended Curettage (N=17)					
	Resection (N=3)	Overall	Electrocautery with Burr (N=2)	Electrocautery with Burr and Phenol (N=2)	Electrocautery with Hydrogen Peroxide (N=4)	Electrocautery with Burr and Hydrogen Peroxide (N=3)	Electrocautery with Hydrogen Peroxide and Ethanol (N=2)
Contractures	0.00 (0.00%)	2.00 (11.76%)	1.00 (50.00%)	1.00 (50.00%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)
Implant Irritation	0.00 (0.00%)	2.00 (11.76%)	1.00 (50.00%)	0.00 (0.00%)	0.00 (0.00%)	1.00 (33.33%)	0.00 (0.00%)
Osteoarthritic Changes	0.00 (0.00%)	2.00 (11.76%)	0.00 (0.00%)	0.00 (0.00%)	1.00 (25.00%)	1.00 (33.33%)	0.00 (0.00%)
Infection	1.00 (33.33%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)
Iatrogenic Fracture	0.00 (0.00%)	1.00 (5.88%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	1.00 (50.00%)
Athrofibrosis	0.00 (0.00%)	1.00 (5.88%)	1.00 (50.00%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)
Recurrence (N=2)	0.00 (0.00%)	2.00 (11.76%)	0.00 (0.00%)	0.00 (0.00%)	1.00 (25.00%)	0.00 (0.00%)	1.00 (50.00%)

Values are presented as frequency (percentage).

Table 5. Descriptive Statistics of the Prevalence of Complications and Recurrence among the Respondents according to Use of Fillers (N = 17)

Complications (N=8)	Bone cement (N=6)	Overall use of femoral head allograft (N=8)	Femoral head allograft with bone cement (N=3)	Femoral head allograft with autograft (N=3)	Femoral head allograft with bone cement and demineralized bone matrix (N=1)
Contractures	1.00 (12.50%)	1.00 (12.50%)	1.00 (33.30%)	0.00 (0.00%)	0.00 (0.00%)
Implant Irritation	0.00 (0.00%)	2.00 (25.00%)	1.00 (33.30%)	1.00 (33.30%)	0.00 (0.00%)
Osteoarthritic Changes	0.00 (0.00%)	2.00 (25.00%)	1.00 (33.30%)	1.00 (33.30%)	0.00 (0.00%)
Infection	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)
Iatrogenic Fracture	0.00 (0.00%)	1.00 (12.50%)	1.00 (33.30%)	0.00 (0.00%)	0.00 (0.00%)
Athrofibrosis	0.00 (0.00%)	1.00 (12.50%)	1.00 (33.30%)	0.00 (0.00%)	0.00 (0.00%)
Recurrence (N=2)	0.00 (0.00%)	2.00 (25%)	0.00 (0.00%)	1.00 (33.33%)	1.00 (100.00%)

Values are presented as frequency (percentage).

lesion that is well-defined and with non-sclerotic margin and an eccentric, mostly epiphyseal location, extending to the subchondral bone, but may often times present with more aggressive features, ranging from a wide zone of transition with cortical thinning and expansile remodeling to cortical bone destruction [1,3,6,16]. The Campanacci grading was created to classify GCTB based on their radiographic appearance and has been proposed to guide treatment [3,5]. Type I are considered latent and are represented by small lesions which are well-defined and with an intact cortex, for which there is more room for conservative management. Type II (Figure 1A) are considered active and relatively well-defined, described as typically larger than type I lesions but with an intact periosteum. Type III (Figure 2A) are aggressive lesions, with indistinct borders, extending through the periosteum and surrounding tissues [3]. In our study, 45% of the respondents had grade II and 55% had grade III GCTB lesions. It has been suggested that grade II lesions should be treated with intralesional curettage and grade III lesions with resection and reconstruction [5]. However, this recommendation has not been incorporated into guidelines, as there is lack of correlation between the grade and the aggressiveness of the GCT and does not provide reliable prognostic significance in terms of recurrence and functional outcomes [5,20]. Further, a study done by Omlor et al last 2019 likewise shows that

Campanacci grade, as well as soft tissue infiltration, and larger size of the lesion does not significantly impact the recurrence of GCTB [15].

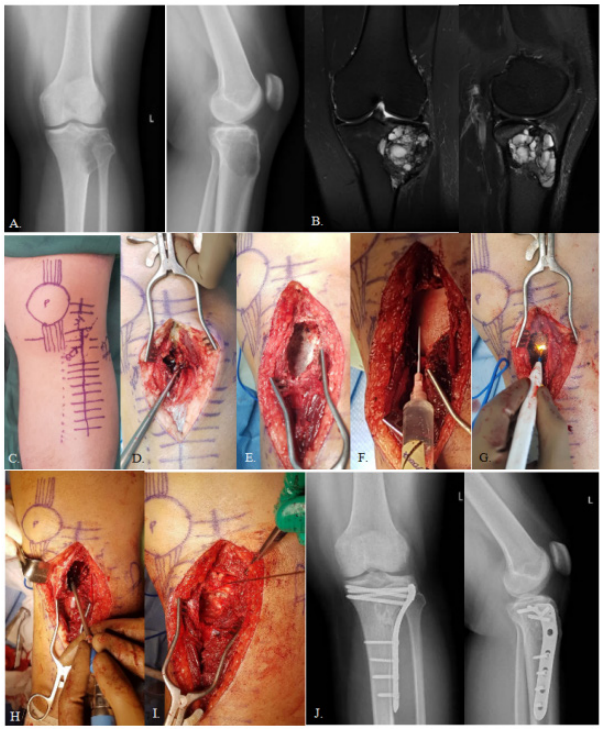

Figure 1. This demonstrates a case done under extended curettage. Preoperative radiographs (A) and MRI (B) reveal a Campanacci II GCTB of the proximal tibia and patient underwent surgery. Surgical markings (C) were done to map the landmarks, area of the lesion, and the planned incision. Curettage (D) was performed revealing grayish, friable tissue. Extended curettage followed using ethanol (E), hydrogen peroxide (F), electrocautery (G), and use of a highspeed burr (H). Bone grafting (I) was done using autologous iliac bone graft adjacent to the articular surface and morselized femoral head allograft to fill the rest of the defect. Postoperative radiographs (J) show placement of a 4-hole L-plate to the medial aspect of the proximal tibia, augmenting the construct.

Due to the wide range of differential diagnoses that can mimic GCTB in imaging, the diagnosis is ascertained only after a biopsy is obtained. The protocol and goal of the biopsy follows as in any other bone tumor. Only upon histopathologic reading of GCTB has been confirmed may surgical planning and treatment proceed [2,3,5]. Surgery is the standard treatment for GCTB, and depending on the involvement of the articulating surfaces, the tumor can be removed either by resection or curettage, with or without adjuvants [2,4-6,14,15] There is still controversy about the surgical management of choice, however the therapeutic goals of surgery remains the same: to achieve a balance between maximizing the removal of the tumor while reducing recurrence rate, preserving function, and preventing occurrence of complications [9,15].

Curettage alone has been the opted treatment of GCTB, due to its ability to provide less morbidity and functional impairment, but regardless how thoroughly curettage is performed, it is associated with local recurrence rates as high as 65% [5,15]. Wide resection (Figure 3), on the other hand, is associated with decreased risk of local recurrence approaching 0% compared to intralesional curettage [2,19]. Despite this however, it is associated with greater surgical morbidity and disability problems leading to poorer functional outcomes [3,14,22]. A retrospective cohort study done by Jamshidi et al investigating the outcomes of patients with Campanacci grade III GCTB of the knee who underwent either RS or EC showed that function was significantly better in the EC group in comparison to the RS counterpart [18]. This further supports the findings in our study, showing good outcomes in those that underwent RS (MSTS 19.67, SD=11.02) in contrast to those that underwent EC who had excellent outcomes (MSTS score 28.18, SD=7.51). Some studies attribute this to curettage being less invasive as well as being able to preserve the joint adjacent to the tumor [15,20]. In contrast, resection results in gradual development of mechanical and structural difficulties in the prostheses of the knee joint, thus affecting the outcomes of these patients in the long term [9,10,14,18,20]. A long-term study by Houdek et al investigating the effects of prosthesis on periarticular tumors of the distal femur reports the same findings and found a high risk of revision and reoperation [11]. This was likewise reported in the study of Jamishidi that showed significantly more revision surgeries in those that underwent RS [18].

Further, evidence shows that 97% of recurrences will occur within two years, with a recurrence after three years being considered exceptional [2,3,14]. As mentioned, high local recurrence rate in curettage without local adjuncts was already described in previous studies [5,15,19]. Hence, EC (Figure 1 and 2) or the addition of local adjuvants such as cryoablation with liquid nitrogen, alcohol, phenol, ethanol (Figure 1E), zolendronic acid, hydrogen peroxide (Figure 1F), electrocautery (Figure 1G), speed burr drilling (Figure 1H), and bone cement (Figure 2H) and combinations of these in addition to curettage to eradicate additional tumor cells has shown to decrease the recurrence in general to 6-25% [3-6]. This is in concordance with our study, with a recurrence of 11.76% (2/17) among those who underwent EC.

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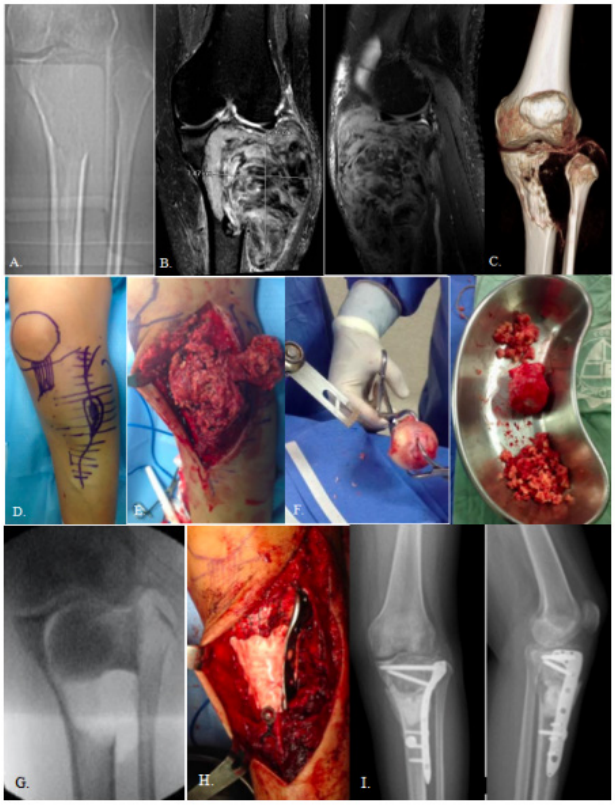
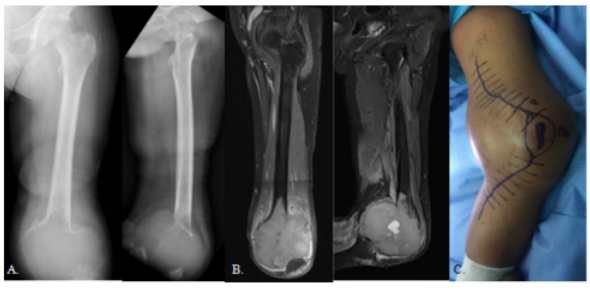


Figure 2. This demonstrates another case utilizing a different technique of extended curettage. Preoperative radiographs (A), MRI (B), and CT scan (C) reveal now a Campanacci III GCTB proximal tibia and patient underwent surgery. Surgical markings (D) were likewise done. Complete exposure (E), and extended curettage proceeded with the use of adjuvants including hydrogen peroxide and electrocautery in 3 cycles. Two femoral heads were secured. One was prepared by removal all surrounding cortical bone and cartilage using a saw to shape it according to the subchondral defect adjacent to the articular surface (G). Plating was then done and patellar tendon was reconstructed and anchored to a post and washer screwed into the tibial shaft; bone cement was then added to augment the fixation (H). Postoperative radiographs (I) show the final construct.



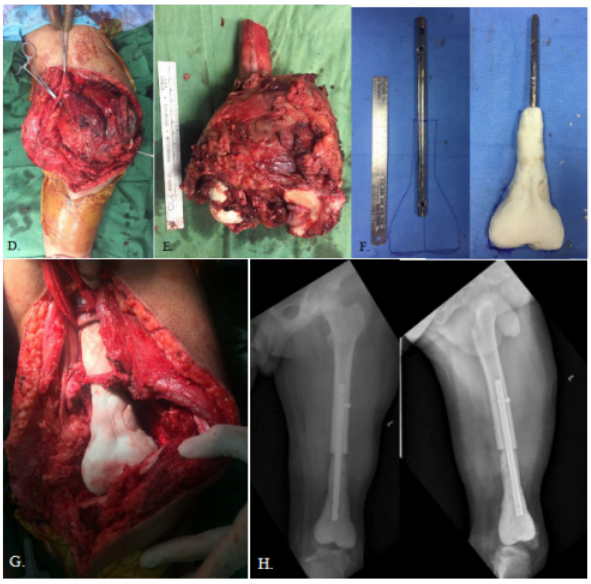


Figure 3. This demonstrates a resection case. Preoperative radiographs (A) and MRI (B) reveal a Campanacci III GCTB of the distal femur and patient underwent surgery. Surgical markings (C) were done to map the landmarks, area of the lesion, and the planned incision. Complete exposure (D) and distal femoral resection were done, revealing a mass measuring 11x10x10cm. Measurements of the femoral shaft and tibial plateau were done and bone cement spacer was shaped accordingly and attached to the end of an intramedullary nail (F). Nail was then secured in place to the femur using a 26mm proximal screw (G). Postoperative radiographs (H) show the implant in place.

In our study, the most common local adjuvant used to extend curettage was hydrogen peroxide alone, comprising 20% of the adjuvants used, and has been as well used in combination with burr and ethanol in 15% and 10% of cases respectively. This may be due to hydrogen peroxide being cheap and easily available, at the same time, being reported as an alternative for phenol with proven in vitro effects against GCT cells [15]. It is noteworthy, however, that hydrogen peroxide use was also seen in the two documented recurrences in our study. Hydrogen peroxide, as demonstrated by Omlor et al, works by inducing apoptosis of cells, and although it has demonstrated a significantly reduced recurrence rate versus in those not treated with hydrogen peroxide and increased recurrence-free survival rate, recurrence can still occur in as high as 22% [15]. In the same study, it was also demonstrated that hydrogen peroxide use versus no hydrogen peroxide use did not significantly influence functional outcomes.

Likewise, electrocautery was also a commonly used local adjuvant and was noted to be used in combination in all 20 cases in our study, but used alone in only 10%. A study investigating the isolated use of electrocautery as

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a local adjuvant for benign bone tumors showed a recurrence of 20.8% in giant cell tumors [17].

Other local adjuvants used in this study were phenol, ethanol, and burr. Phenol, although is considered historically as the most prevalent chemical adjunct in the treatment of GCTB for its ability to cause cell lysis and death and is able to decrease recurrence rate to 6-18%, was found to cause serious chemical burns and can be systemically toxic, hence have not been as commonly used [19,21]. Ethanol, on the other hand, is more readily available and less toxic as well [21]. A study by Jones et al reports a recurrence rate of 13.5% following primary curettage of GCTB with adjuvant ethanol [21]. Some authors would recommend the use of a burr to help break ridges of bone in order to obtain adequate exposure of the lesion during curettage and improve the thoroughness of tumor removal [14,16]. A study by Balke et al in 2007 mentions that the use of a high-speed burr decreases residual tumor cells in the area by its thermal effect and the likelihood of recurrence was 4 times higher if burring was not done [19]. Despite this, there are reports of a 12% recurrence rate with the use of burrs in combination with EC [2,3].

As these multiple studies portray, these adjuncts tested alone and in combination were proven to reduce the rate of local recurrence, however there always seems to be a risk of recurrence that may be attributed to residual microscopic disease often under pockets or ridges of bone [3,19]. In addition, due to their heterogenous use, their effectivenesss in terms of reducing recurrence has not been proven [15,19]. Hence, recurrence still has to be discussed with patients as possible outcomes of surgery.

In this paper, there were eight cases that utilized femoral head allograft as fillers in defects after curettage was performed. These grafts offer advantages such that it undergoes remodeling and once incorporated, may offer permanent stock and support in a defect [14]. Among these eight cases, the two documented recurrences both had use of these allografts. A study by Sobti et al notes that bone graft reconstruction in addition to curettage alone is only able to afford some local control and may have recurrence rates as high as 50%, and hence is recommended to be used in adjunct to other forms of EC [3]. However, to prevent cartilage destruction, adjuvants to EC such as burring and electrocautery may not be done as aggressively as needed thereby explaining why recurrence might be higher in these cases [19].

On the other hand, the use of polymethylmethacrylate (PMMA) alone was the most common filler used for defects in this study, comprising 35.29%. The advantages of this alternative include its availability, cost, and can offer immediate stability for patients where compliance is of question [16]. It has also been reported that use of PMMA results in local hyperthermia which may induce necrosis of residual disease of up to 3 mm, decreasing recurrence to 10-14% [2,3]. The downside to using bone cement in subchondral regions is that it may damage the adjacent articular cartilage.

A study done by Gao et al investigated functional outcomes in patients treated with bone grafts versus bone cement following curettage in patients with GCT of the long bones. Their study found that MSTS scores were significantly lower than in patients treated with bone graft compared with bone cement. which was contrary to the findings in our study, which reported excellent outcomes for both bone cement and bone allografts. Their study also reported a higher recurrence rate in patients treated with bone grafting. The same findings were found in our study and a study done by Vaishya et al likewise found that recurrence rate was significantly higher in patients treated with bone grafting as compared with patients with PMMA [22]. However, it was recommended that ultimately, extensive curettage be performed extensively with some adjuvant therapy to help in decreasing the incidence of recurrence [22]. In addition, as mentioned in the study of Klenke et al, the selection of bone graft versus cement should always remain individualized [16].

5. Conclusions

There is excellent functional outcome among patients who underwent EC as compared to those who underwent RS as limb salvage surgery for GCTB of the lower extremity. However, there was also note of higher incidence of complications in those who underwent EC over RS. This may indicate that despite the occurrence of complications of the salvaged extremity, this does not necessarily associate with poor outcomes as the patients continue to report good functionality. Nevertheless, it is important to keep in mind that in the face of excellent outcomes, there is still the possibility of recurrence that is associated with EC. Surgeons must hence reiterate the importance of compliance to follow-up to monitor subsequent events. It is recommended that a larger population and a longer follow-up be used in future studies to increase the power and generalizability of the results of this study.

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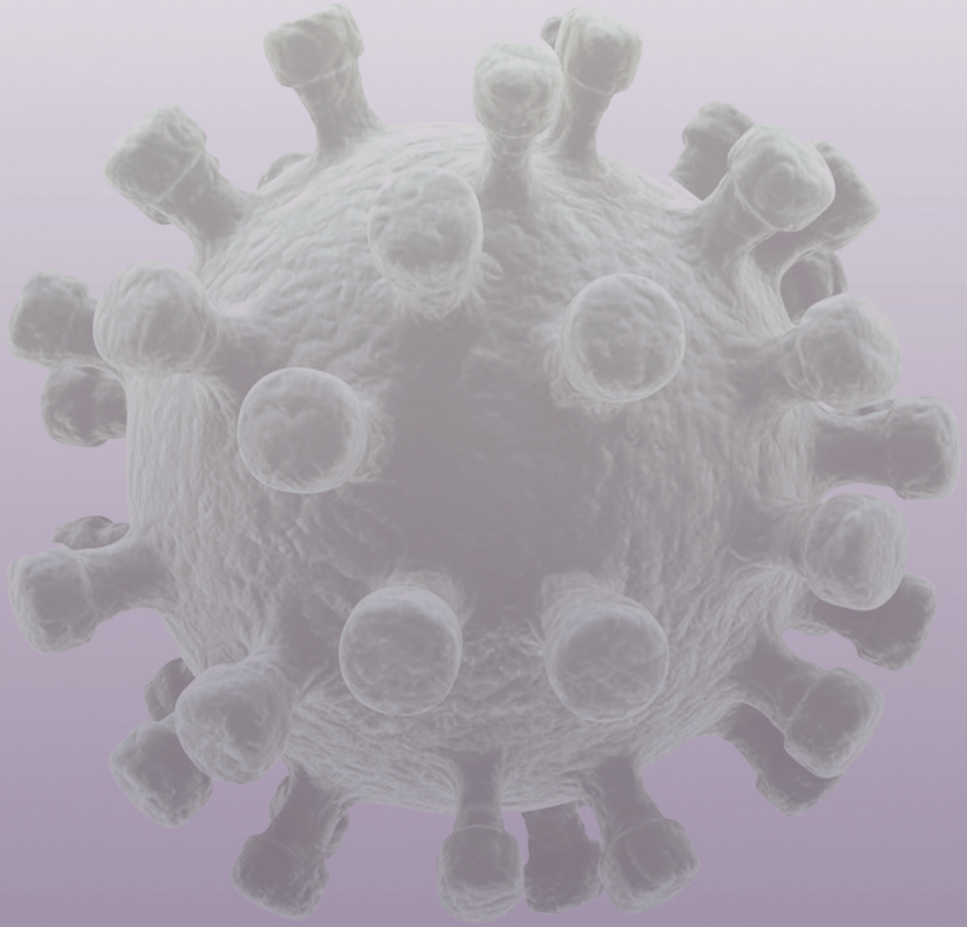

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