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ARTICLE

Measurement of AhR Ligands in the Tissues of Colon Cancer Patients with XRE Luciferase Reporter

Bystriakova Margarita Koshkin Sergei Tolkunova Elena*

Institute of Cytology of the Russian Academy of Science, St-Petersburg 194064, Russia

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ABSTRACT

The Aryl hydrocarbon receptor (AhR) ligands exhibiting modulating activity represents a new class of anticancer agents that can be directed towards several tumors. We have examined AhR expression in human colon cancer and adjacent non-tumor tissue. AhR expression level was about 2-7 times higher in tumor tissue samples than in the adjacent non-tumor samples (in 82% of all the samples). We were unable to find any increase of ABCG2 expression on the level of the transcription, while the expression of MDR2 was increased in half of the tumors compared to the levels of expression in normal adjacent tissue. We have used FICZ as a potent high affinity ligand of the AhR to calibrate the reporter cell line HEK293T-AhR-luc as a potent high affinity ligand of the AhR. The concentration of xenobiotic response element (XRE) ligands is higher, than in the blood of healthy people in 86% of the patients. The proposed test system will allow the use of the AhR ligand level as an additional diagnostic marker in the treatment of colon cancer.

1. Introduction

The presence of polycyclic aromatic hydrocarbons (PAHs) in the environment is a source of concern for specialists in the field of organic chemistry, biochemists, environmental chemists and geochemists. Many PAHs are potent chemical carcinogens [1]. The deterioration of the environmental situation is associated with the increase in cancer incidence, including colorectal cancer. There were approximately 1.4 million new cases of colorectal cancer in 2012, making it the third and second most common cancer globally among men and women, respectively [2].

AhR is an environmental response gene that mediates cellular responses to a variety of xenobiotic compounds that frequently function as AhR ligands. The protein encoded by AhR is a ligand-activated helix-loop-helix transcription factor involved in the regulation of biological responses to PAHs. The AhR that is present in the non-active state is cytosolic. Before ligand binding, AhR is sequestered in the cytoplasm; upon ligand binding, this protein moves to the nucleus and stimulates transcription of target genes after binding to specific DNA sequence elements known as the xenobiotic response elements (XREs). XREs are present in the regulatory region of target genes including xenobiotic-metabolizing enzymes (XMEs) such as members of cytochrome P450 family (CYP1A1, CYP1A2, CYP2B1 and UGT1A6) [3].

Immunostaining of normal intestinal tissue sections

**Corresponding Author:*
Tolkunova Elena,
Institute of Cytology of the Russian Academy of Science, 4 Tikhoretsky avenue, St-Petersburg, 194064, Russia;
E-mail: entolk62@mail.ru

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allows the localization of AhR in the stroma that contains immune cells and lymphoid follicles, whereas in the tumor tissue immunostaining was detected in both stromal and tumor cells. Given that, it can be seen that AhR expression is increased in tumor tissues. Enterocytes of the small intestine have a great ability to detoxify PAH, so that the epithelium of the small intestine is the first barrier to the absorption of PAH. Barrier function is provided by XME and efflux carriers that transport metabolites from the cell. ATP-binding cassette subfamily G member 2 (ABCG2) and P-glycoprotein (ABCB1/MDR2) is known for mediating the efflux of conjugated metabolites of xenobiotics [4-6]. ABCG2 is a constitutively expressed ATP-binding cassette (ABC) transporter that protects many tissues against xenobiotic molecules.

In various cancers ABCG2 transporters are known to produce multidrug resistance (MDR), thereby limiting the clinical response to chemotherapy. They show activity that changes the pharmacokinetics of the drugs used and diminishes the effectiveness of their delivery to tumor cells, causing the formation of multidrug resistance. ABCG2 was also identified as a direct target of AhR [7]. AhR abundantly expressed in many different types of cancer. Thus, AhR pathway on one hand assists in the eradication of PAHs, but, on the other hand, the side effect of its activation is a certain decline of treatment efficiency via the chemotherapeutic agent's efflux.

In recent years, the interest of human AHR research has shifted to the study of the physiological functions of AHR and immunity control [8-10]. Along with its involvement in chemical protection, researchers are most interested in its involvement in providing stem cell homeostasis, as well as in modulating the immune system [11].

The identification of tumorigenic cancer stem cells (CSCs) in colorectal cancer and the mechanism contributing to the formation of their qualities and the maintenance of homeostasis requires additional study and remains unidentified.

Significantly increasing the effectiveness of spheroid formation, resistance to chemotherapy and the ability to form tumor xenografts of choriocarcinoma cells after activation of AhR by TCDD has been reported recently. Expression of ABCG2 is also related to tumorigenic CSC in several cancers. The possible role of AhR in CSC quality maintenance is highly interesting to the application of AhR as a marker of CSC and can be used for creating a target drug.

Based on the studies in recent decades, researchers have come to understand the causal role of FCs in several human diseases. The role of AhR as a diagnostic marker and a possible therapeutic target in the treatment of these pathologies is suggested [12].

There are reporter constructions that allow to estimate the activity of the main signaling pathways under the control of the responsive elements to various nuclear receptors by the level of the luciferase expression. Although technically not a member of the Nuclear Receptor superfamily, the AhR shares many of the same attributes. To study the changes in AhR activity we have decided to use XRE luciferase reporter. Our reporter construct is similar to the plasmid from the well-known chemically activated luciferase expression (CALUX) system [13] that has been used for the rapid and inexpensive detection and relative quantitation of dioxin-like chemicals in a wide variety of sample matrices. Although the Caco-2 adenocarcinoma cell line is traditionally used as a model to study the intestinal epithelium, we have decided to use HEK293 cells to make a xenobiotic responsive cell line. The goal of our study was to create a reporter cell line, which will make it possible to estimate xenobiotic concentration in a serum of colorectal cancer patients and in tumor tissue. HEK293 cell line was used for transfection with the reporter construct, which contained the luciferase gene under control of a XRE repeated 6 times and a minimal promotor. The level of Luc expression, measured in the relative units, reflects the concentration of PAHs in the culture media of reporter cells line. To calibrate the reporter cell line we had used FICZ as a potent high affinity ligand of the AhR.

2. Materials and Methods

2.1 Cell Culture

Experiments were done with HEK293T cell line and reporter cell line HEK293T-AhR-luc. The cells were maintained in a DMEM medium (Biolot) with 10% bovine embryonic serum (Gibco), penicillin and streptomycin (Biolot). The cells that have reached the monolayer were subcultured in the ratio of 1:3.

2.2 Tumor and Adjacent Mucosa Samples

Colon adenocarcinoma, adjacent normal tissue and blood serum from 11 patients were used in experiments, these patients were ranked according to the severity and extent of the oncological process, according to the clinico-morphological (TNM) classification (Table 1). Also blood serum from healthy people was used.

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Table 1. Clinico-pathological characteristics of patients

Patient	Sex	Age	TNM*	Tumor localization
P1	F	67	$pT_{is}N_0M_0G_1$	Transversum colon
P2	F	66	$pT_4N_0M_0G_2$	Transversum colon
P3	F	84	$pT_4N_0M_0G_2$	Transversum colon
P4	M	66	$pT_3N_0M_0G_1$	Transversum colon
P5	M	70	$pT_3N_0M_0G_2$	Transversum colon
P6	F	68	$pT_3N_0M_0G_2$	Cecum
			$pT_3N_1M_0G_2$	Transversum colon
P7	M	63	$pT_3N_1M_0G_2$	Cecum
P8	M	66	$pT_3N_1M_0G_3$	Transversum colon
P9	F	72	$pT_3N_1M_0G_3$	Transversum colon
P10	M	49	$pT_3N_1M_1G_2$ (hepar)	Transversum colon
P11	F	56	$pT_3N_1M_1G_2$ (hepar)	Transversum colon

Note: Main clinico-pathological information of patients. The position in the table was determined based on the clinical prognosis coming from the pTNM classification and additional prognostic markers (level of tumor differentiation, tumor grow rate). F- female, M- male. pTNM - Classification system of the anatomical extent of cancer. p - stage given by histopathologic examination of surgical sample. T - size of primary tumor, N - degree of spread to regional lymph nodes. M – present of distant metastasis and site.

2.3 RNA Isolation, Synthesis of cDNA and qPCR

Total RNA was isolated from cultured cells and tissue samples (with preliminary homogenization) using the RNeasy Mini Kit (Qiagen). cDNA was generated from 1 µg of total RNA per sample using the RT M-MuLV–RH kit (Biolabmix, Russia). qPCR was performed by using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and the HS-qPCR SYBR Blue kit (Biolabmix, Russia). GAPDH and B2M genes were used to normalize gene expression. The results are represented as a fold induction using the ΔΔCt method.

2.4 Cloning of XRE and Map of the Reporter Plasmid

The plasmid vector pGL4.27(luc2P/minP/Hygro) was used for XRE cloning. The vector contains a multiple cloning region for insertion of a response element of interest upstream of a minimal promoter and the luc2P gene. The vector contains an ampicillin resistance gene to allow the selection in E. coli and a mammalian selectable marker for hygromycin resistance. KpnI and HindIII sites of the polylinker region were used for restriction and ligation during the preparation of XRE vector under a minimal promoter. The thrice repeated 21 bp XRE was used as an insert. Synthetic oligonucleotides has KpnI and HindIII sites on the ends and after annealing were ready for ligation into the restricted vector. The oligonucleotides sequences are presented below:

F-XRE/KpnI-HindIII (65 bp)

ctg agt tct cac gct agc aga ttg agt tct cac gct agc aga ttg agt tct cac gct agc aga ta

R - XRE/HindIII-KpnI (73 bp)

agc tta tct gct agc gtg aga act caa tct gct agc gtg aga act caa tct gct agc gtg aga act cag gta c

2.5 Reporter Cell line Creation

After the transfection of a plasmid reporter into HEK293T cells with Lipofectamit 2000, clones with integrated construct were selected into the culture media, containing 100 mkg/ml Hygromycin B. Several clones were selected and their response to AhR ligand inducible activation of luciferase expression was checked out. One clone was chosen and used in the presented study as HEK293T-AhR-luc cell line.

To calibrate the reporter cell line we have used FICZ (Merck) as a potent high affinity ligand of the AhR. We used ligand solution in concentration from 100nM to 0.032 nM, because this reporter line was created for working in the range of nearly physiological concentration of PAHs. Luciferase assay was performed by using the Luciferase Assay Systems (Promega). Cell viability was determined with MTT assay after 24 h.

We have determined the expression level of AhR and its surrogate target CYP1A in a reporter cell line in response to the treatment of FICZ. 7. FORMATTING Please, make the figures bigger. Our studies clearly show an mRNA increase for CYP1A in response to the FICZ (0.8 nM) treatment of the reporter line cells.

2.6 Luc-assay

Increased luciferase activity was used to estimate the AhR activation, induced by the components in a culture medium. 1.5×10^4 cells per well were seeded in a 96-well tissue culture plate in a standard growth medium, 24 hours prior to the test. On the day of the experiment the media was replaced with a fresh growth medium containing 10% heat inactivated patient's blood serum or lysate of patient's tissues. For the preparation of colon adenocarcinoma and adjacent normal tissue

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lysates 25 mg of each samples were taken and placed in 500 mkl of buffer solution (20 mM Tris pH 7.6; 100 mM MaCl; 5 mM MgCl2; 1 mM EDTA). Then the samples were disrupted and homogenized with the pestle, underwent ultrasound treatment (3 times, 15 sec each impulse in 80% of maximal intensity) and centrifuged for 3 min at maximum speed. The exposure within luciferase assay buffer and substrate was performed by using the protocol for Luciferase Assay Systems (Promega). The cytotoxicity of the compounds screened against the HEK293T-AhR-luc cell line was tested in parallel by measuring the cell viability using MTT-assay. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU's) per 100 000 cells. Viability of cells was measured by a colorimetric method using MTTassay. Optical density was measured at a wavelength of 570 nm against a solution of MTT with DMSO on a Multiskan EX spectrophotometer (Thermo Electron, USA). The amount of unsoluble formazan correlates with the number of viable cells in the population.

2.7 Statistics

RNA level and cell viability were evaluated after three identical tests. Statistical difference was calculated in the analysis of variance using Statistica 6.0. $p<0.05$, which was considered to be statistically significant. Mixed-model analysis of variance (ANOVA) or the Student's t test was used to analyze data from the luciferase reporter assays, and P values less than 0.05 were considered as statistically significant.

3. Results

3.1 AhR Expression in Human Colon Cancer

We examined AhR expression in human colon cancer and adjacent non-tumor tissue (figure. 1A). RT-PCR analysis has revealed that the AhR expression level was about 2-7 times higher in tumor tissue samples than in adjacent non-tumor samples (in 9 from 11 samples). For one of patient we have registered a couple hundred-fold of an increase in the level of expression of AhR by qPCR. We have observed the increased AhR expression in tumor cells, however the level of expression was not connected with the stage of the disease.

The results of a comparative analysis of the level of AhR mRNA in groups of patients suffering from different types of cancer were published. The data shows that in the tumor tissue of patients with cancer of the thyroid gland, pancreas, stomach or colon, the mRNA content in the tumor tissue is higher compared to non-tumor tissue. However, as noted by Safe et al. (2013), AhR mRNA levels were not predictive for patient survival [14].

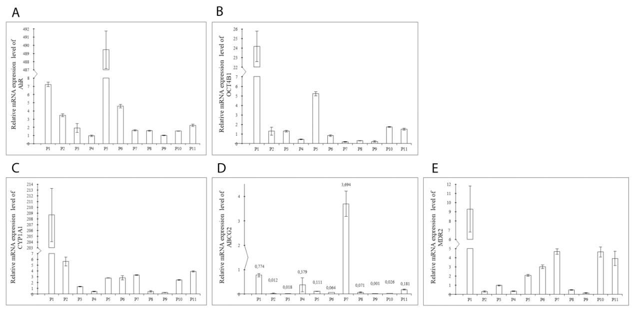

Figure. 1. mRNA expression in patient tissues.

P1-11 - patients with colorectal cancer. From the left to the right, patients are represented by the increased stage of disease according to the classification of TNM staging system. For each patient mRNA level in normal tissue was taken as 1. mRNA expression level is shown in the tumor tissue relative to normal tissue. Height of column above 1 indicates overexpression, below — of its supression. (A) Relative level of AhR mRNA. (B) OCT4B1 mRNA. (C) CYP1A1 mRNA. (D) ABCG2 mRNA. (E) MDR2 mRNA.

We have compared the levels of excess expression of AhR levels in tumor tissue compared to normal tissue with expression levels of OCT4B1, a potential marker of colon cancer stem cells (figure 1B). We have found that in the samples expressing OCT4B1 at an elevated level compared to the adjacent normal tissue, the level of expression of AhR was also increased. We assume that there is a link between the expression level of AhR and the enrichment of the tumor population with stem-like cells potential.

3.2 Expression of CYP1A1 and ABC Family Members in Human Colon Cancer

The biotransformation of potentially toxic chemicals occurs in two distinct phases, Phase I and Phase II, and involves several enzyme systems, the most important being the P450 cytochromes. We have compared the levels of expression of CYP1A1 in tumor samples and adjacent tissues and registered a 2-5-fold increase in 8 samples out of 12 (figure 1C). To eliminate toxins, the body has developed several transporter systems, such as the P-glycoprotein, which prevents the absorption of chemicals through the gastrointestinal tract by facilitating their efflux from the enterocytes into the intestinal lumen [15]. We were unable to find any increase of ABCG2 (figure 1D) expression on the level of tran-

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scription, while expression of MDR2 (figure 1E) was increased in half of the tumors compared to the levels of expression in normal adjacent tissue. No dependencies of the expression levels of the efflux transporters of the tumor malignancy stage were found.

3.3 Calibration Curve for Reporter Cell Line

To calibrate the reporter cell line we have used FICZ as a potent high affinity ligand of the aryl hydrocarbon receptor (AhR). A descriptive statistics analysis of HEK293T-AhR-luc calibration curve was performed using Excel (Microsoft). In this range of concentrations calibration curve can be described by the following equation $y=12.99*x0.19$, where y (RLU's) is the luciferase activity at ligand, (FICZ) is the concentration d (nM) (figure 2A). The coefficient of determination $R=0.19$ so the model can be considered quite good [16]. A calibration curve allows to determine the concentration of PAH in the tissues of the given patients, converts the Relative Light Units (RLU's) to the concentration of PAH (nM). We consider the change in level of luciferase expression in response to the binding of the PAHs mixture in the sample to be equivalent to the effect of the FICZ. Luciferase activity was correlated to the number of viable cells determined by the MTT method.

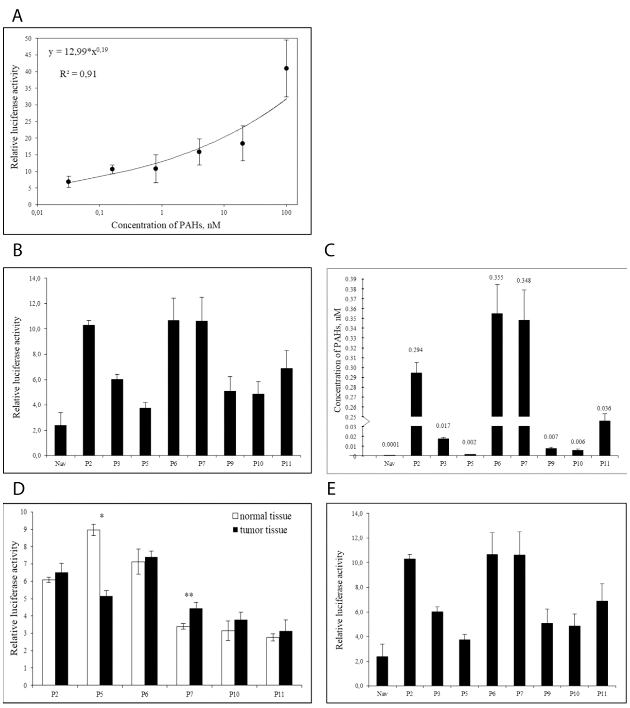

Figure 2. Measurement of AhR ligands (PAHs) concentration.

Note: (A) Calibration curve for reporter cell line HEK293T-AhR-luc. P2-10 - patients with colorectal cancer. Nav (average norm) – average for the blood of 6 healthy people. (B) Concentration of PAHs in blood of patients in RLUs. (C) Concentration of PAHs in blood of patients in nM. (D) Concentration of PAHs in a surgical sample of cancerous and normal tissue in RLUs. (E) Concentration of PAHs in a surgical sample of cancerous and normal tissue in nM. n=3, p<0.05.

3.4 The Concentration of XRE Ligands in the Blood of Patients with Colon Cancer

It is known that the defeat of the human body by xenobiotics most often and most effectively occurs through food chains (up to 96% of the PAH enters the human body with food). To answer the question whether the presence of colon cancer correlates with the content of AhR ligands in the blood serum, we have compared the effect of the blood serum of sick and healthy people on the level of luciferase expression when cells were added to the culture medium of HEK293T-AhR-luc cells. The increase in luciferase expression level (RLU's) reflects the amount of PAHs in the blood of patients with CRC. By using the calibration curve we have determined the concentration of PAHs in nM. As it can be seen from the figure (figure 2B and 2C), for 6 out of 7 patients, the number of PAHs is higher than in the blood of healthy people. We believe that there is a correlation between the disease and the level of AhR ligands in the blood. It is impossible to determine whether a tumor produces endogenous ligands or this increase is due to the influx of PAHs from the outside. On the basis of the small group of samples, we cannot reach a final conclusion about the difference in the content of PAH in the blood, however, we assume that their level in patients with colorectal cancer is higher than that in healthy people.

3.5 The Concentration of XRE Ligands in the Tumor and Normal Tissue of Patients with Colon Cancer

The next question was to compare how the tumor transformation affects the level of ligands in the colon. For this, the effect of lysates of tumor tissue and adjacent normal mucosa on the level of luciferase expression was compared by adding the lysates into the culture medium of HEK293T-AhR-luc cells (figure 2D and E). It is clear, that the amount of AhR ligands is gets lower with increasing malignancy. However significant discrepancy between the level of luciferase activity in tumor tissue and the normal mucosa of the same patient was not registered in 4 out of 6 cases, whereas another two patients demonstrated a different character of dependency. We cannot prove the effect of tumor transformation on accumulation of PAHs in tumor tissues. Comparison of the levels of ligands in the normal mu-

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cosa of patients with CRC has shown high heterogeneity (up to 3 times). We are unable to explain the nature and causes of this heterogeneity due to small cohort of patients.

4. Discussion

Huge progress has been made in the study of AhR signaling and the identification of new endogenous ligands, including the high-affinity ligand FICZ and kinurenin, both of which are tryptophan metabolites [11]. However, extensive studies of human AhR are required, since the functions of the receptor differ both in different species and in cells of different tissues, and also depending on the cellular environment [17]. Ligands of AhR not only have different affinities, but also a different nature - endogenous and exogenous, and can both activate and inhibit the receptor. The proposed reporter allows us to determine only the total concentration of the ligands of the AhR, there is no possibility to separate endogenous and exogenous ligands. That is, the activity of luciferase expression will reflect the level of activation of the AhR signaling pathway in response to the entire spectrum of ligands and allows us to observe the dynamics of the effect of therapy. The quantitative determination of the PAH by the method of mass spectroscopy in one sample reaches $ 1,000 and requires complex and complicated sample preparation. In this sense, the use of the reporter line repeatedly simplifies and reduces the cost of analysis, making it possible to make it accessible and widely used in both bio-medical and environmental studies. Cultivation of reporter cell line does not require expensive multicomponent media, the analysis is simple to do in many replications, and when cultivated, the cells are sufficiently resistant to the addition of the test samples to the culture medium. In order to check the activation of xenobiotic-metabolizing enzymes as a response to ligand binding to XRE, we have determined the expression level of AhR and its CYP1A surrogate target in reporter cell line. Our studies show an mRNA increase for CYP1A after FICZ (0.8 nM) treatment, while levels of AhR expression were similar (data not shown). However, as indicated above, a small number of samples does not allow making statistically reliable conclusions about differences in gene expression levels for patients with colorectal cancer compared with healthy ones. Therefore, the reporter strain can be used to test the content of PAH in various materials - water, milk, extracts of meat, fish, juices of vegetables and fruits, as well as in biological fluids - saliva, blood, urine.

We observe a significant increase of PAH level in the serum of patients with CRC independent of stage. We believe that we were the first to reveal such a difference in the levels of AhR ligands using a simple luciferase analysis. A similar pattern of differences was observed in the analysis of tissue lysates of tumors and adjacent mucosa - in 5 of 6 patients we have noted an increase of the PAH from 2-6 times. Proposed system does not allow to determine the origin of ligands (accumulation of endogenous or the deposition of exogenous).

Recognition of the potential for therapeutic use of AhR ligands required considerable experimental data and a long time to be confirmed due to well-known genotoxicity [14]. In recent reviews there is increasing evidence that the AhR and its ligands can be used as targets in the development of new drugs for antitumoral therapy [11, 14, 18].

The development of drugs that targeted AhR must take into account the selectivity property of modulators, manifested in the fact that the receptor ligand will serve as AhR agonist or antagonist depending on the tissue context [14, 19].

Thus, since it is known that AhR agonists enhance the growth of colon and stomach cancer cells, there is an assumption about the possible therapeutic role of selective modulators exhibiting antagonistic activity in this cellular context. In contrast, in most pancreatic tumors PAH inhibit cancer cell proliferation and anchorage-independent growth [20], suggesting that selective agonists will be effective for treating pancreatic cancer.

HEK293T-AhR-luc test system will allow to study the relationship between the concentration of PAHs and the degree of tumor malignancy (stage, tumor size, the presence of metastases) and serves for development of new selective modulators of AhR for cancer chemotherapy.

Conflicts of interest

The authors indicate no potential conflict of interests regarding the publication of this paper.

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Publication ethics

The paper received the ethical approval of St. Petersburg clinical scientific and practical center of specialized types of medical care (oncological) Ethics Committee.

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