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Examination of Telomerase Expression with Immuno-Hystochemistry Techniques on Some of Cancer Cells

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Abstract

Background: Cancer is a disease that gets serious attention in the medical world. This is due to the ever increasing number of patients and there has been no effective way to treat. Cancer cells have telomerase activity is relatively high compared to normal cells, so the cancer cells have the ability to continue to proliferate. Cancer cells undergo uncontrolled mitosis and have high telomerase activity compared to cells normal. Telomerase is an enzyme responsible for telomere length, a segment of DNA that is the tip of chromosomes in eukaryotic cells. Telomeres are associated with the process of ageing and carcinogenesis. The purpose of this study was to determine the expression of telomerase in some cells such as breast cancer, cervical cancer, and lung cancer. **Methods:** The research method is experimental studies in several cancer cell cultures in the form of cell line. Cancer cells used were: HeLa (cervical cancer), MCF7 and T47D (breast cancer), WiDr (lung cancer), and Raji (lymphoma) with culture medium RPMI, DMEM, and M199. Vero cells is used (fibroblast cells) as a control (normal cells). Expression of telomerase enzyme was measured by the Immunohystochemistry (IHC) method. **Results:** The results showed that the cancer cells have activity/higher telomerase expression were highly significant ($p < 0.01$) compared to normal cells (Vero cells). Similarly, the expression of telomerase in HeLa versus WiDr, WiDr versus T47D, T47D versus Raji, and Raji versus MCF7 also showed highly significant differences ($p < 0.01$). Telomerase expression between cancer cells that showed significant difference (HeLa cells versus Raji cells; HeLa cells versus MCF7 cell; T47D cells versus MCF7 cells) ($p < 0.05$). No significant difference was found in the group of HeLa cells versus T47D, WiDr versus Raji cells, and WiDr versus MCF7. **Conclusions:** It was concluded, that the cancer cells have telomerase expression of specific and different from each other, depending on the type of cell. T47D breast cancer cells have telomerase expression of the highest, followed by cervical cancer cells (HeLa). Lung cancer cells (WiDr) with cell lymphoma (Raji) has almost the same expression and both have lower expression.

Keywords: HeLa, IHC, MCF7, telomerase, WiDr

Introduction

Cancer is a process that occurs gradually include initiation (irreversible genetic changes) and promotion (population of cells which extends initiation) and eventually malignancy. It is known that the enzyme telomerase plays a role in every cell replication that is able to maintain long telomeres to offspring, so that when the cancer cells do not have sufficient telomerase enzyme, then the growth of cancer cells is stalled by itself.[1,2] Of the several types of cancer, cervical cancer remains the most common cancer among women. It is the second major cause of women's death in Asia as well as in Indonesia.[3]

On the other side are known, genes contained in the DNA molecule of a chromosome in the cell nucleus. Segments of DNA on the ends of chromosomes known as telomeres and is one of the factors related to the occurrence of cancer. Telomeres consist of highly specific nucleotide sequence, the human sequence is TTAGGG is repeated hundreds or even thousands of times, so the formula is the nucleotide structure of telomeres (TTAGGG) n. T, A, and G show the nucleotide (DNA-forming compound collection) which contains thymin, adenine, and guanine bases. In humans there are 2.000 repetitions on the base unit. In one organism in different cell types, any number of repetitions of different nucleotides.[1,4] The length of telomeres play an important role in maintaining genome stability and regulate replication and cell death. In length or short telomere is maintained by the enzyme telomerase, which serves to lengthen telomeres and DNA damage repair. The exact shape of this enzyme may different from one species to another species, but each version has a specific template RNA subunit to form a new telomere.[5,6] Telomerase activity in normal cells is mainly

found in the reproductive cells because these cells have unlimited proliferative power. Telomerase consists of two functional components, namely the telomerase reverse transcriptase (TERT, hTERT in humans) and telomerase RNA template.[7,8] Telomerase activity is mainly regulated by hTERT gene that plays an important role in the growth and development of tumours through the maintenance of telomere length.[8]

The presence of the enzyme telomerase in a variety of cancer cells each may give different expressions depending on the type or location of the cancer cell and telomerase is not found on normal cells. It can be used as the basis of treatment of cancer cells if there are materials/substances that can inhibit the activity of the enzyme telomerase. Cancer cells have a high mitotic activity because cancer cells undergo uncontrolled mitosis and have higher telomerase activity compared to normal cells. There are no reports or data more thoroughly on the enzyme telomerase expression in several different types of cancer cells. The purpose of this study was to determine the expression of telomerase in several types of cancer cells such as breast cancer, cervical cancer, and lung cancer by in vitro.

Methods

In this research, the enzyme telomerase expression was measured in cultured cancer cells (cell line) from breast cancer, cervical cancer, lung cancer, and lymphoma. The study consisted of six groups consisting of one group of normal cells (Vero) as control and five other groups are cancer cells (T47D, HeLa, WiDr, Raji, and MCF7). Telomerase expression was measured by using a technique Immunohystochemistry (IHC) and calculated the percentage of cells which showed positive expression.

Materials. Materials used in this study were breast cancer cell line (T47D and MCF7), cervical cancer cell line (HeLa), lung cancer cell line (WiDr), and lymphoma cancer cell line (Raji). Also used a normal cell line (Vero) as a comparison. Whole cell line was obtained from the collection of the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta. The medium used is the medium Roswell Park Memorial Institute (RPMI) 1640 (Sigma) for cell Raji, T47D, HeLa, and WiDr; media Dulbecco's Minimum Essential Medium (DMEM) (Gibco) for cells MCF7 and medium M-199 (Sigma) for Vero cells. The materials for IHC are Fetal Bovine Serum (FBS) (Gibco BRL), Phosphate Buffer Saline (PBS), Sterptomisin penicillin (Merck), telomerase antibody (Sigma) (Dako Corporation), HEPES, SDS (Sodium Dodecyl Sulphate) (Dako Corporation). The tools used in the study include CO_2 incubator (Inc 2 Memmert), autoclave (Cryolab 35), laminair cabinet irflow (LAF) (Lab Konko), microscope (Olympus), and tissue culture flasks (Nunclone).

Preparation of Culture Media and Growing of Cells. Preparation of culture medium RPMI (Rosewell Park Memorial Institute) is to dissolve the powder to one liter of RPMI 1640 into akubides approximately 800 mL, plus 2 g of sodium bicarbonate and HEPES 2 g, was added to one liter of distilled water. The solution was stirred with a magnetic stirrer about 10 minutes until homogeneous, then neutralised with 1 N HCl to pH 7.2 to 7.4. Media of cells growing made by mixing FBS as much as 19 mL, 2 mL Streptomycin penicillin, Fungizone 0.5 mL, then diluted using RPMI 1640 culture medium to 100 mL. Furthermore, the solution was filtered with a filter polyethene sulfone in aseptic sterile 0.2 µm. Stored in the refrigerator by using a sealed bottle.

Telomerase expression examination by Immunohystochemistry (IHC). Cell culture T47D, MCF7, HeLa, WiDr, Raji and Vero with density 10^4 orwell already attached to the coverslip performed immunohistochemical staining (IHC) in the laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta. Measurement procedure telomerase expression with IHC method, as follows: the cell culture coverslip that had been treated were incubated at 5% CO_2 incubator, temperature of 37 °C for 24 hours, taken and placed on a glass object (poly-l-lysine slides), fixation with acetone or methanol for 10 minutes at -20 °C. Then washed with PBS 3 x 5 min, then dropped 0.3% H_2O_2 for 20 minutes. After that spilled with normal mouse serum (1:50) for 15 minutes, the liquid discharged (without washing), then sprinkled with primary antibody (telomerase) for 60 minutes. Wash in PBS 3 times each for 5 minutes, then incubated in secondary antibody biotin (biotinylated secondary antibody) for 5-10 minutes, washed with PBS 3 times each for 5 minutes. Incubation in streptavidin-peroxidase enzyme for 5-10 minutes, washed with PBS 3 times each for 5 minutes. Tetrahidrochloride deamino-benzidine incubation in chromogen (DAB) for 5-10 minutes with chromogen substrate ratio of 1:20, washed with distilled water. Preparations immersed in hematoxylin for 3-5 minutes to counterstain, washed with distilled water and dehydrated performed using 95% ethanol, then with xylene each for 10 minutes. Further preparations spilled mounting media (Canada balsam) and covered with a glass cover. Telomerase expression was observed using a light microscope. Cells that express telomerase will give a brown color, whereas cells that did not express the will give a purplish blue color.

Results

Calculation of telomerase expression by IHC method is done by counting the number of cells that showed positive expression (brown nucleus and cytoplasm) of 100 cells were examined. Calculations performed with 3 samples. The results are expressed in percent. Percentage of positive expression values are presented in Table 1.

The percentage of positive telomerase expression in cancer cells showed high expression. ANOVA test results showed the highly significant difference ($p < 0.01$) between groups of cancer cells with normal cells or the control group (Vero cells), with a value of F = 178.489 (Table 2).

The expression of telomerase in cancer cells in this study, including breast cancer cells, cervical cancer, lung cancer, and lymphoma showed higher expression than normal fibroblast cells (Vero). Besides the magnitude of the expression of telomerase in cancer cells, respectively, differ from each other. The results of the multiple comparison test LSD (Least Significant Difference) between each group is presented in Table 3. Telomerase expression in all groups of cancer cells (HeLa, WiDr, T47D, MCF7, and Raji) results showed highly significant difference ($p < 0.01$) than the control group (Vero). In addition it is also showed highly significant difference ($p < 0.01$) among the group of cancer cells as between HeLa versus WiDr, WiDr versus T47D, Rajiv versus T47D, and MCF7 versus Raji. Telomerase expression was significant difference ($p < 0.05$) was found between HeLa versus Raji, HeLa versus MCF7, T47D versus MCF7, while between HeLa versus T47D, WDR versus Raji, and WiDr versus MCF7 showed no significant difference ($p > 0.05$).

Discussion

Telomerase is an ribonucleoprotein enzyme that maintains protective structure at the ends of eucaryotic chromosomes, called telomeres. Telomerase is a structure that is responsible for the maintenance of telomeres, a replication of nucleotides (TTAGGG) n. In most human somatic cells, for example fibroblasts, suppressed the expression of telomerase and telomeres shorten progressively at each cell division. In contrast, most of the tumor cells or human cancer cells expressing telomerase, resulting in telomere length is stable. These observations suggest that telomere maintenance is essential for the proliferation of tumor cells or cancer.[9,10] Telomerase activity determine cell proliferation, both cancer cells and normal cells under conditions in vitro and in vivo.[11,12]

Although telomerase activity in cancer cells increased their activity, but De Lange reported that cancer cells telomeres are shorter than the surrounding normal tissue. The researchers speculate that telomerase becomes activated after cells lose their ability to proliferate. Telomerase activity in the cancer cells will increase after a certain phase is when the cell loses some substance telomeric. Finally, the enzyme telomerase is activated to stabilize telomere, the cancer cells will be immortal cell, and there was a continuous cell proliferation.[1] Telomerase plays a

Table 1. The Average Value of the Percentage of Positive Telomerase Expression from Each Group

No	Control Group (Vero)	HeLa Group	WiDr Group	Raji Group	T47D Group	MCF7 Group
1	5	77	74	67	86	74
2	5	78	64	67	90	74
3	3	90	64	67	90	73
Mean	4.33	81.67	67.33	67.00	88.67	73.67
SD	1.15	7.23	5.77	0.00	2.31	0.58

Table 2. Anova Results of the Expression of Telomerase in between Groups

	Sum of square	Df	Mean of square	F	Sig
Between groups	13781.778	5	2756.356	178.469	0.000
Within groups	185.333	12	15.444		
Total	13967.111	17			

Table 3. Test of Multiple Comparisons (LSD) Percentage of Positive Telomerase Expression between Each Group

Group	Control group (Vero)	HeLa	WiDr	Raji	T47D	MCF7
Control (Vero)		**	**	**	**	**
HeLa			**	*	-	*
WiDr				-	**	-
Raji					**	**
T47D						*
MCF7						-

*significant different at $p < 0.05$
**there are very significant difference at $p < 0.01$

key in cell immortality and tumorogenesis. Telomerase is activated in 80-90% of human cancer cells and it is not found in normal somatic cells so it can be used as a marker in the diagnosis of cancer. Clinical trials for telomerase can be useful as a non-invasive method for the early detection and monitoring of cancer.[13,14]

Most normal cells or somatic cells such as fibroblasts (Vero cells) have a limited ability to replicate is generally 40 to 50 generations, then will stop dividing, because the normal cell undergo aging and apoptosis. This is determined by the presence of erosions or telomere shortening, since most normal human cells lacking telomerase activity in the presence of strict repression of transcription of human telomerase reverse transcriptase (hTERT). In normal somatic cells telomere shortening, including stem cells, which are intended for cell renewal. So a somatic cell has the aging program.[5,7,12,15] The above results indicate that, despite the expression of telomerase in cancer cells is much higher than the normal cells, but in each of the cancer cells are not the same expression. Telomerase expression is different in each species and each type of cell will show different telomerase expression, as did the level of develop-ment.[12,16,17]

Breast cancer cells showed the highest expression than other cancer cells. Previous research on breast cancer reported that telomerase activity is regulated by human telomerase reverse transcriptase gene (hTERT), which plays an important role in the growth and development of breast cancer cells (MCF-7 and MDA-MB-231) through the maintenance of telomere length.[8] Whereas telomerase expression in normal cells have been found in the germ cells in the ovaries and testes, and stem cells. Whereas in adult somatic cells telomerase expression is very low or does not show any expression of telomerase.[18] The enzyme telomerase plays a role in every cell replication that is able to maintain telomere length to cell derivatives, so that if cancer cells do not have sufficient telomerase enzyme, then the growth of cancer cells by itself is discontinued when the enzyme is no longer able to sustain long continuous telomere due to abnormal proliferation control. The absence of the enzyme telomerase can inhibit the growth of cancer cell division by transmitting so that shorter telomeres and die before making any further damage. However, if the cancer cells are able to synthesise telomerase that cancer cells will continue to proliferate and become immortal.[12,19]

From the description and the results above showed that telomerase is active or high prevalence in cancer cells, so that telomerase can be an attractive therapeutic target. In recent years, many standard therapeutic agents used to treat cancer that acts as an anti-telomerase. Such as in breast cancer has been to development tamoxifen, trog-litazone as anticancer agents that are anti-telomerase.[6,20] On the other hand is known, that cyclooksigenase enzyme-2 (COX-2) may play a role as well as a specific marker that could help determine the diagnosis of cancer. Over-expression of COX-2 plays an important role in the carcinogenesis process. It has been reported that the expression of COX-2 increased in some cancer cells, such as lung cancer and breast cancer. COX-2 enzyme may increase cancer cell proliferation and resistance to apoptosis and suppression of COX-2 will cause apoptosis.[21] Suppression of hTERT expression in cancer cells will also improve the process of telomere shortening, thus triggering cell senescence and apoptosis. The target telomerase or hTERT as a strategy in anticancer therapy, is relatively new. Inhibition of telomerase or hTERT alone has minimal clinical efficacy. Has conducted research for the targeting of treatment through a combination of telomerase or hTERT and COX-2 to work synergistically in killing cancer cells.[7] In addition to the target in therapy, telomerase is also used as an attractive target for the diagnosis and prognosis of breast cancer. The results of the examination of telomerase may be a marker of breast cancer diagnostics and improve diagnostic accuracy. Estimates of telomerase activity in various variants of breast cancer may predict the clinical course of the disease and provide prognostic instructions for different variants of breast cancer.[22]

Conclusions

Cancer cells have telomerase expression of specific and different from each other, depending on the type of cell. T47D breast cancer cells have telomerase expression of the highest, followed by cervical cancer cells (HeLa). Lung cancer cells (WiDr) with cell lymphoma (Raji) has almost the same expression and both have lower expression.

Conflicts of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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