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Real-Time Expression of hTERT in Primary Melanoma Biopsies

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A B S T R A C T

Skin melanoma is by far the most lethal skin cancer, it is unpredictable by nature and presents a severe diagnostic problem. One of the major issues in melanoma diagnostics is to differentiate it with confidence from a dysplastic nevus. Thus, the aim of this study was to evaluate hTERT expression on a spectrum of dermal lesions (from normal skin to primary melanoma) in order to examine its possible role as a diagnostic marker in melanoma diagnostics. In this study we analyzed the expression of hTERT by real-time PCR on 58 freshly obtained biopsy samples (4 samples of normal skin, 12 dermal nevi, 23 dysplastic nevi, 19 primary melanomas). Our results showed slightly greater hTERT expression in dysplastic nevi than melanomas with major data overlap. Considering the given results, hTERT does not seem to be a reliable diagnostic marker for melanoma.

Key words: *hTERT, primary melanoma, Real-Time, expression, biopsies*

Introduction

Telomerase is an enzyme that synthesizes telomeric repeat sequences (TTAGGG)n, which protects chromosomes from shortening that consequently leads to recombination, end-to-end fusion and rearrangements[1]. Cells without telomerase activity exhibit limited growth ability whereas cells showing telomerase activity are immortalized. Telomerase is a ribonucleoprotein complex consisting of two subunits: the human telomerase RNA (hTR), which is complementary to the telomeric single stranded overhang and is used as a template for the synthesis of TTAGGG repeats and the human telomerase reverse transcriptase (hTERT), catalytic protein subunit which is responsible for the process of telomeric DNA synthesis[2]. Telomerase activity is the most general molecular marker for the identification of human cancer and can be detected in 85% of all tumors, whereas most healthy tissues exhibit little or no telomerase expression. The hTR is ubiquitously expressed in humans, whereas hTERT mRNA expression is suppressed in most somatic tissues after neonatal period[3]. Considering that expression of hTERT is strongly correlated with telomerase activity in skin tumors, we decided to measure hTERT expression[4]. Malignant melanoma accounts for less than 5% of all skin cancers but it is by far more lethal. The incidence and mortality rates of malignant melanoma have been rapidly increasing in the last decade[5,6].

The most difficult issue in the melanoma diagnostics is clear differentiation between melanoma and a dysplastic nevus. There are no clear guidelines to recognize dysplastic nevi that show most potential towards malignant transformation. Furthermore, there is a need for a more detailed prognosis and follow-up management.

In this study we evaluated the expression of hTERT mRNA on a freshly obtained dermal tissue using real-time PCR. We attempted to examine expression levels between dermal, dysplastic nevi and malignant melanoma showing different Clark-Breslow staging and associate them with pathohistological diagnostic/prognostic parameters.

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Materials and Methods

Patients and tissues

In total, 58 freshly obtained samples (4 normal skin, 12 dermal nevi, 23 dysplastic nevi, 19 primary melanomas) were examined for hTERT mRNA expression. Samples were obtained from the patients that were admitted to the Department of Surgery, Rijeka University Hospital Center, for lesion removal and were diagnosed at the Department of Pathology, School of Medicine, University of Rijeka, in 2009. All patients were informed about procedures to be conducted and upon understanding and agreeing they signed a written consent. Excised biopsy samples were immediately immersed and stored in RNA later (Takara Bio inc., Otsu, Shiga, Japan) and were subsequently taken to the dermatopathologist for microscopic evaluation. The dermatopathologist first inspected excised samples on a snap frozen section and determined if there was enough material to perform routine diagnostics and conduct a study. A small portion of designated samples selected for the RNA isolation were stored in RNA later at –30°C for two weeks until the patients were given the complete diagnosis.

RNA isolation

Tissue samples were homogenized in MagNA Lyser (Roche, Basel, Switzerland) and RNA was isolated according to the manufacturer instructions using Nucleospin RNA isolation kit (Machery-Nagel, Düren, Germany). Purity of RNA was evaluated using Cary 100 UV-Vis spectrophotometer (Varian Inc, USA). Only samples with 260/280 nm ratio between 1,6–1,9 were used for further analysis.

cDNA synthesis

Isolated RNA (0,8–1,2 μg) was supplemented with DEPC treated H_20 to reach the final volume of 8μL. PrimeScript Reverse transcriptase Kit(Takara Bio inc., Otsu, Japan) was used for reverse transcription. Oligo dT primers were selected to eliminate unnecessary ribosomal RNA and cDNA was scanned on Cary 100 UV-Vis spectrophotometer(Varian Inc., Palo Alto, USA) to get the final concentration of the material used in the real time PCR reaction.

Real time quantitative PCR

Quantitative, real-time PCR has been described earlier by Heid et al.[7]. Briefly, 2μL of cDNA was used for both hTERT and β-2-microglobulin (reference gene) expression measurement in tissue samples. Standard curves which were made for hTERT and β-2-microglobulin (Figure 1) showed efficiency under 1.9 and error under 0.25 which was the prerequisite for further data analysis. Reactions were run on a LightCycler 1.5 Instrument (Roche, Basel, Switzerland) with LightCyclerSoftware 4.05 using four step cycling program that consisted of the following: 1) Denaturation-1cycle; 2) Amplification 45 cycles 95°C-10sec, 55°C-20 sec, 72°C-20 sec. Sybr Green Premix Ex Taq (Takara Bio inc., Otsu, Shiga, Japan) was used for real time PCR reaction. Primers for hTERT can be found in Primer Bank (PrimerBank ID 22759946a2). Sensors collected emission spectra of all samples on 530nm channel during the last 20 seconds of the primer annealing/elongation step. The reaction was set to 40 cycles after which melting point analysis and relative quantification were made.

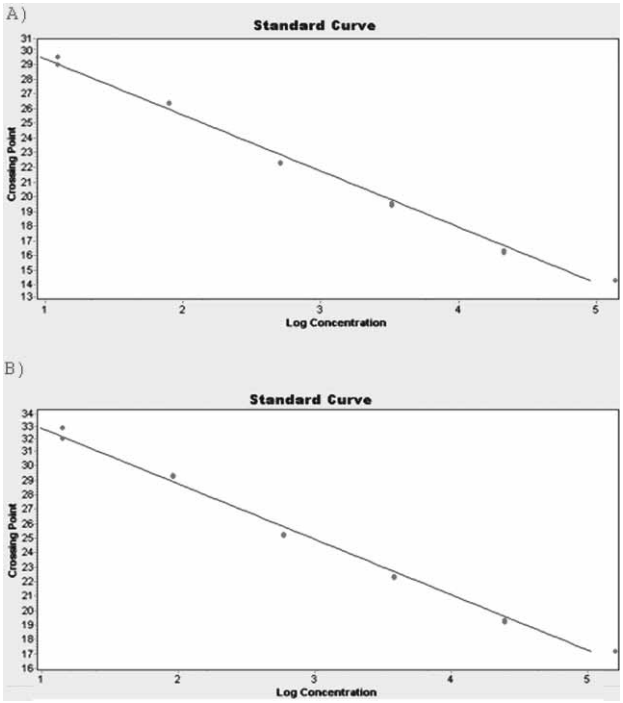

Fig. 1: A) β-2microglobulin standard curve (Error: 0.195 Efficiency: 1.818); B) hTERT standard curve (Error: 0.232, Efficiency: 1.827). TA – Telomerase Activity, β2M – β-2-microglobulin.

Results

The level of expression of hTERT is presented in Table 1. Expression of hTERT was identified in almost all samples. All nevi showed hTERT expression but also 4 out of 12 samples of normal skin. The normal skin which was used as a control was non UV-irradiated. The expression level varied between samples but nevertheless it showed distinct pattern. In general, dermal nevi showed

TABLE 1
AVERAGE hTERT RATIO NORMALIZED FOR B2M HOUSEKEEPING GENE

Mean hTERT relative ratio	[1000x(hTERT/β2M)]	Standard deviation
Normal skin	0.92x10	±0.235
Dermal nevi	$81x10^2$	$\pm3.25x10^2$
Dysplastic nevi	$22x10^4$	$1.25x10^4$
Primary melanoma	$92x10^3$	$2.57x10^3$

expression similar to the expression of normal skin while dysplastic and melanocytic lesions showed elevated expression. To our surprise, hTERT levels in dysplastic nevi were somewhat higher than those measured in melanocytic lesions.

Discussion

Major problems in melanoma diagnostics are dysplastic nevus management and progression evaluation. It is mainly due the fact that malignant melanoma has shown to have large heterogeneity in molecular pathogenesis. Several studies have tried to establish molecular markers as prognostic and/or diagnostic markers in malignant melanoma. Most markers were connected with dysregulated growth factor signaling and tyrosine kinase inhibitors[8]. However, none of them showed to be valid and reliable.

TA (telomerase activity) has been detected in almost all human cancers, including breast, ovary, bladder prostate, stomach, colon, liver, brain, and skin cancers, and, at lower levels, in some preneoplastic lesions, whereas, it is repressed in most somatic cells[9]. There have been several studies trying to evaluate TA in melanoma. Studies were conducted on paraffin embedded tissues and in vitro cells. However, a study using fresh clinical material was needed to obtain more relevant data. Concerning the afore mentioned correlation between TA and hTERT expression real-time PCR measurement of hTERT expression would provide a more precise insight than outdated immunohistochemical methods measuring TA. The results showing that in this data pool dysplastic nevi have higher hTERT expression than malignant melanoma are unexpected and contradictory to some former studies concerning TA and melanoma[10]. All prior research showing higher TA or hTERT expression in melanomas than dysplastic nevi include overlapping ranges[11]. In this study benign and malignant type also showed overlapping expression range. However, dysplastic nevi had somewhat greater hTERT expression than primary melanoma.

Our findings are not in agreement with previous studies. This could be explained by a small total sample count, but it can also be explained by the nature of telomerase. For a gene to be defined as an oncogene, it needs to have a direct impact on the loss of cell growth control. Telomerase is responsible for cell immortalization but there must be a different trigger for the loss of growth control, so, telomerase is not an oncogene. Immortalizing cells facilitate the accumulation of mutations[12]. Thus, immortal cells have more chance to gather the critical mutation which will trigger cancer formation.

We believe that telomerase cannot be considered a reliable diagnostic factor which was also concluded by some other authors[11]. Different methods provide different results that cannot be widely applicable. However, hTERT and telomerase are enzymes with many functions which are still undiscovered and need further research.

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REAL-TIME EKSPRESIJA HTERT-A U BIOPSIJAMA PRIMARNOG MELANOMA

SAŽETAK

Melanom kože je najsmrtonosniji među tumorima kože, po prirodi je nepredvidiv te predstavlja ozbiljan dijagnostički problem. Razlikovanje displastičnog nevusa od malignog melanoma je jedna od najvažnijih stavki u dijagnostici

melanoma. Cilj ove studije jest evaluacija nivoa ekspresije hTERT-a u različitim dermalnim lezijama (od normalne kože do malignog melanoma) te procjena učinkovitosti hTERT-a kao biomarkera u dijagnostici melanoma. U ovoj studiji smo analizirali ekspresiju hTERT-a koristeći Real-time PCR u 58 bioptata svježeg tkiva (4 uzorka normalne kože,12 dermalnih nevusa, 23 displastična nevusa, 19 primarnih melanoma).Naši rezultati pokazuju nešto veću hTERT ekspresiju u displastičnim nevusima nego u melanomima uz veliko preklapanje podataka. S obzirom na dobivene rezultate hTERT se nije pokazao kao dobar biomarker u dijagnostici melanoma.