Characterization of Telomerase (*hTERT*) in Solid and Hematopoietic Cancer Cell Lines Reveals Different Expression Patterns

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Abstract. *Background/aim:* Overexpression of human telomerase reverse transcriptase (*hTERT*) allows disordered proliferation and immortality of malignant cells, which has been of interest for the development of targeted therapies. The present study aimed to characterize *hTERT* gene expression in a series of cancer cell lines. *Materials and Methods:* Leukemia cell lines K-562, its vincristine-resistant derivative K-562-Lucena1 and daunorubicin-resistant derivative FEPS; gastric adenocarcinoma lines AGP01, ACP02 and ACP03; melanoma SK-Mel-103 cells; and MN01 and MRC5, two non-neoplastic cell lines were analyzed by real-time polymerase chain reaction in order to evaluate *hTERT* gene expression. *Results:* In leukemia cells, *hTERT* gene expression was significantly increased only in K-562 ($p<0.05$) and K-562-Lucena1 ($p<0.001$) when compared to the calibrator MRC5. For solid tumor types, only ACP03 presented a significant *hTERT* gene expression when compared to ACP02 ($p<0.05$). *hTERT* gene expression in K-562 and K-562-Lucena1 was significantly increased ($p<0.05$ to $p<0.001$) compared to all other cell lines except ACP03. *Conclusion:* In leukemia cell lines, *hTERT* gene overexpression was shown to be a potential target for pharmacological assays for drugs aiming to inhibit telomerase activity and control cell proliferation in oncohematological diseases.

Cancer cells have properties that maintain them in sustained proliferative activity, with a striking pattern known as

replicative immortality, which is one of the most important characteristics and problems in the fight against these cells in the organism of affected species. Therefore, the ability of cells to replicate in a disorderly way and not undergo senescence has been shown by tumor models to be due to telomerase overexpression (1).

Human telomerase reverse transcriptase (*hTERT*) is an enzyme responsible for the maintenance of chromosomal ends and, in turn, protects DNA from degradation, being expressed in about 85% of cancer cells and poorly expressed in healthy cells (2-5).

Inactivation or inhibition of the *hTERT* gene has been used as a means of restraining the replication of neoplastic cells. In this context, the expression of telomerase in most types of cancer cells has attracted the interest of studies towards development of targeted therapies (6, 7).

Experimental oncology is a branch of science that uses established human cell lines with original genetic and morphological characteristics of the tissue of origin as tools for understanding the biology and molecular pathways (genetic and epigenetic) of different tumor types for the subsequent development of new therapies in order to characterize malignant tumor patterns in basic research. In order for these studies to be increasingly objective and illuminating, the characterization of these cell lines is necessary, especially for the most important purpose of experimental oncology, which is the development of new antineoplastic drugs, in addition to understanding the mechanisms of action of and resistance or sensitivity to new chemotherapeutics and those already used in the treatment of cancer (8-10).

However, studies point to variation in gene expression between the same cell lines grown in different laboratories. It has been suggested that the variation is caused by epigenetic mechanisms, such as methylation, and due to

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Table I. Cell lines evaluated in this study.

Cell line	Tissue of origin	Classification	Reference
AGP01	Ascitic fluid of gastric adenocarcinoma	Intestinal	14
ACP02	Gastric antrum adenocarcinoma	Diffuse	14
ACP03	Gastric cardia adenocarcinoma	Intestinal	14
SKMel-103	Melanocyte	NA	15
MN01	Non-neoplastic gastric epithelium	Normal cells	16
K-562	Bone marrow chronic myeloid leukemia	NA	17
K562-Lucena1	Derived from K-562	Vincristine-resistant	13
FEPS	Derived from K-562	Daunorubicin-resistant	19
MRC-5	Human fetal lung fibroblasts	Normal cells	

NA: Not applicable.

different culture conditions from one laboratory to another, and variation in the gene expression of these lines modifies the original morphology and proliferation (10, 11).

The aim of this study was to characterize *hTERT* gene expression in a panel of tumor cell lines usually used in experimental oncology in order to validate the models as potential screening tools for target-directed therapies against telomerase activity in cancer.

Materials and Methods

Cell culture. For the evaluation of *hTERT* gene expression, a panel of experimental oncology lines was used. The chronic myeloid leukemia cell lines K-562, vincristine-resistant derivative K-562-Lucena1, and daunorubicin-resistant derivative FEPS were kindly provided by Professor Vivian M. Rumjanek from Federal University of Rio de Janeiro, Brazil (12, 13) and maintained in RPMI medium. The K-562-Lucena1 and FEPS cell lines were maintained with RPMI medium supplemented with 60 nM vincristine sulphate and 46 nM daunorubicin, respectively. The gastric adenocarcinoma cell lines AGP01, ACP02 and ACP03; melanoma cell line SK-Mel-103; and two non-tumoral cell lineages from gastric epithelium (MN01) and lung fibroblast (MRC-5) were maintained in Dulbecco's modified Eagle's medium. All cell lines were maintained in media with specific supplements and incubated with 5% CO₂ at 37°C (Table I).

Extraction of RNA and reverse transcription of RNA to DNA. RNA from all cell lines was extracted with TRIzol Reagent® (Invitrogen™) according to the manufacturer's instructions. From 20 ng of RNA, the cDNA was synthesized using High Capacity cDNA Reverse Transcriptase kit (Life Technologies, Carlsbad, CA, USA) to convert the extracted and purified RNA to cDNA. The conversion step was performed on a Veriti® thermal cycler (Applied Biosystems®, Foster City, CA, USA). After this step, the samples were stored in a freezer at -20°C until use for analysis.

Validation of gene expression by real-time quantitative polymerase chain reaction (qPCR). The gene selected for evaluation of telomerase expression was *hTERT* (Hs_00972650_m1), and the gene *GAPDH* (Hs02786624_g1) was used as an internal control.

The detection method was the TaqMan® Gene expression assays system (Applied Biosystems and qPCR was performed using QuantStudio5 Real-Time PCR system (Applied Biosystems For each sample, the following were used: 3 µl of cDNA, 1 µl of each primer/probe, 12.5 µl of TaqMan® Gene Expression Master Mix (Life Technologies, Carlsbad, CA, USA) and 8.5 µl of ultra-pure water. The gene-expression levels were based on absolute and relative analyses and calculated using the 2^{-ΔΔCT} (delta delta threshold cycle) method (19) using the MRC-5 cell line as the calibrator/control. Each sample was analyzed in triplicate for the validation of the technique and the values of CT, according to the international standards for evaluation of gene expression by real-time PCR (20).

Statistical analysis. Analysis of one-way variance (ANOVA) was used to analyze gene-expression data, with an acceptable significance level of 5% (*p*<0.05). Test data were analyzed from the mean and standard deviation of three independent experiments. The Bonferroni correction method was used for multiple comparisons.

Results

In the comparative analysis of leukemia cell lines, *hTERT* gene expression of the K-562 strain was significantly increased compared to that of MRC-5 (*p*<0.05), as it was in K-562-Lucena1 cell line (*p*<0.001). However, in this analysis, there was no statistical significance (*p*>0.05) in the difference of expression of *hTERT* in FEPS compared to the calibrator MRC-5. Leukemia cell lines K-562, K-562-Lucena1 and FEPS, when compared among themselves, did not differ statistically significantly (Figure 1).

Regarding comparative analysis of the gastric adenocarcinoma, melanoma and normal gastric epithelial cell lines with MRC-5, the results showed that there was only significant expression of *hTERT* in the ACP03 cell line (*p*<0.05), and that expression of *hTERT* in ACP03 was significantly greater when compared with ACP02 (*p*<0.05) (Figure 2). Our results also show that the MN01 normal gastric epithelial cells had similar expression of *hTERT* gene to the AGP01 and ACP02 gastric adenocarcinoma cell lines.

Figure 1. Evaluation of human telomerase reverse transcriptase (*hTERT*) gene expression in leukemia cell lines. Fold change data are represented as mean $\pm$ standard deviation of three independent experiments. *hTERT* gene expression was compared between leukemia cell line K-562, its vincristine-resistant derivative K-562-Lucena1, daunorubicin-resistant derivative FEPS, relative to that in MRC-5 lung fibroblasts, using ANOVA statistical test and multiple Bonferroni comparisons. Significantly different compared to FEPS at * p <0.05 and ** p <0.001.

Finally, we compared *hTERT* gene expression between all the cell lines. Our results show K-562 cells to have significantly higher *hTERT* expression in relation to MN01 (p <0.05), AGP01 (p <0.05), ACP02 (p <0.001) and SK-Mel-103 (p <0.05) cells. The K-562-Lucena1 cell line presented significantly higher gene expression in relation to MN01 (p <0.001), AGP01 (p <0.001), ACP02 (p <0.001) and SK-Mel-103 (p <0.001) cells (Figure 3).

Discussion

In differentiated human cells, telomerase is silenced due to inhibition of the *hTERT* gene. However, in malignancies, telomerase is highly activated, being essential during oncogenesis for stabilizing telomere length and in order to confer replicative immortality capacity on the cell, as well as confer resistance to chemotherapy and radiotherapy (20).

Mutations in the promoter of the *hTERT* gene are considered the most common genetic alterations in various types of cancer (21-23). On the other hand, there are tumor types that not have a high frequency of mutations in the promoter region of this gene. In this context, telomerase has attracted the interest of studies that involve the inactivation or inhibition of its activity, as a means of restricting cellular proliferation in cancer, inducing the re-entry of the cell into

Figure 2. Evaluation of human telomerase reverse transcriptase (*hTERT*) gene expression in solid tumor cell lines: gastric adenocarcinoma (AGP01, ACP02, ACP03), melanoma (SKMel-103) and non-tumoral gastric mucosal (MN01) cells. Fold change data are represented as mean $\pm$ standard deviation of three independent experiments. Comparison of *hTERT* gene expression between gastric adenocarcinoma, normal gastric epithelial cells relative to that in MRC-5 lung fibroblasts was made using ANOVA statistical test and multiple Bonferroni comparisons. ***Significantly different compared to all other cell lines at p <0.001.

Figure 3. Comparison of human telomerase reverse transcriptase (*hTERT*) expression between leukemic cell lines (K-562, vincristine-resistant derivative K-562-Lucena1, and daunorubicin-resistant derivative FEPS), gastric adenocarcinoma, (AGP01, ACP02, ACP03), melanoma (SKMel-103) and non-tumoral gastric mucosal (MN01) cells. Fold change data are represented as mean $\pm$ standard deviation of three independent experiments. Comparison of *hTERT* gene expression was made using ANOVA statistical test and multiple Bonferroni comparisons. Significantly different at * p <0.05, ** p <0.001 and *** p <0.0001 when compared to normal cell line MRC5.

the normal cell cycle and, consequently, leading to programmed cell death, known as apoptosis (3, 7, 24, 25).

hTERT overexpression in K-562 and K-562-Lucena1 cell lines may be related to mutations in the promoter of the gene as shown in the study of Lansdorp *et al.* (26), who describes that *hTERT* amplification may also be seen as a cellular response to a decrease in telomeres size, considering that they tend to decrease in size in leukemia. Literature also shows that telomeres are shorter in leukemia cells with cytogenetic alterations, and shortening of telomeres is an important marker of cell malignancy (27-29). Therefore, shortening of the ends of the hematopoietic cell chromosomes may increase the chance of translocations and chromosomal loss, leading to genomic instability and cytogenetic changes (28).

With regards to the results for the AGP01, ACP02, ACP03, MN01, SK-Mel-103 and MRC-5 cell lines, it was noted that ACP03, an intestinal gastric cancer type according to the Lauren classification, had high *hTERT* expression compared with the ACP02 diffuse-type cell line ($p < 0.05$). In a study by Choi *et al.*, patients with gastric cancer who had neoplastic diffuse cells had shorter telomeres, while patients with neoplastic intestinal lesions did not present telomere shortening. The authors further point out that a polymorphism in the *hTERT* gene may increase gene expression in intestinal-like cells (30). Therefore, we suggest that the differential expression of *hTERT* in the ACP03 cell line may be related to a polymorphism in the coding region of the gene.

Gastric cancer of the diffuse type has a worse prognosis when compared to the intestinal type (30). The prognosis of patients with gastric cancer can be determined by tumor size and diameter (31). Altered expression of *hTERT* in gastric adenocarcinoma cells contributes to the invasive and metastatic potential of these cells (32, 33). In this context, overexpression of *hTERT* in the ACP03 cell line may allow the cell to be more capable of tissue invasion, giving it the characteristic of greater aggressiveness when compared to the other gastric adenocarcinoma cell lines.

It is important to emphasize that the microenvironment in which a cancer cell is found is an important factor in the positive or negative regulation of this gene. According to Wang *et al.*, the high expression of the *hTERT* gene in gastric adenocarcinoma may be related to physiological factors, such as acidification of bile acid, which induce hyperexpression of telomerase through the activation of the MYC gene, promoting tumor progression (34, 35).

When evaluating the expression of *hTERT* in the melanoma cell line SK-Mel-103, there was no statistical difference compared to the other strains, including that of normal gastric epithelium (MN01). Vicecont *et al.* showed that some melanoma cell line, do not express *hTERT* but, when it expressed, this is related to mutations in the promoter region of the gene (36).

When we compared all the cell lines used in this study, the leukemia lines (K-562 and K-562-Lucena1) showed high expression of the *hTERT* gene in comparison to gastric adenocarcinoma (AGP01, ACP02), melanoma (SK-Mel-103) and the normal gastric epithelial (MN01) cell lines. These results can be explained by the stimulus for telomerase expression that leukemia cells receive in order to compensate the shortening of the telomeres present in this type of cancer. Chromosomal translocations caused by shortening of the telomeres promote an increase in the expression of *hTERT* gene, and these phenomena are described in leukemia and other oncohematological diseases (26-28, 37), while in gastric adenocarcinoma and melanoma, point mutations or polymorphisms are main factors that increase *hTERT* gene expression (30, 36).

Zhang *et al.* found telomerase activity to be associated with cell lineage differentiation: the lower the telomerase activity, the more differentiated was the cell lineage; and the more differentiated, the lower was the replicative capacity of the cell (7). Based on this, the K-562 and K-562-Lucena1 cell lines would be expected to have a larger replicative capacity than AGP01, ACP02, SK-Mel-103 and MN01 because of their high expression of *hTERT*.

Finally, expression of *hTERT* may have been affected by changes in splicing of the *hTERT* gene (38,39). In addition, there is the possibility that the *hTERT* gene has functions independent of maintenance of telomeres (26).

Conclusion

The overexpression of *hTERT* in leukemia cell lines, compared to solid tumor cell lines, indicates that, in these cell lines, *hTERT* may be a target for pharmacological assays aiming to inhibit telomerase activity and control cell proliferation in oncohematological diseases. The tumor type of origin of the ACP03 line, by overexpressing *hTERT*, may be highly invasive and aggressive, and this cell line might also be useful in pharmacological trials of telomerase-targeting therapies. However, studies measuring the size of telomeres are necessary to understand the relationship of *hTERT* expression with the maintenance of telomeres in the studied cell lines.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Author's Contributions

Moreira-Nunes CA, Mesquita FP, Portilho AJS and Montenegro RC performed the study design; Holanda LS and Portilho AJS performed the cell culture analysis; Holanda LS, Mesquita FP, Portilho AJS and Moreira-Nunes CA performed the molecular and statistical analyses; Holanda LS, Moraes-Filho MO, Moraes MEA,

Montenegro RC and Moreira-Nunes CA wrote the article. All Authors read and approved the final article.

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