

Relationship between dyskerin expression and telomerase activity in human breast cancer

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Abstract. The nucleolar protein dyskerin is involved in the modification of specific uridine residues to pseudouridine on ribosomal and small nuclear RNAs and in the stabilization of the telomerase RNA component (TERC). In this study we investigated for the first time the relationship between dyskerin expression and telomerase activity in a series of 61 primary breast carcinomas. We found that when dyskerin mRNA values were very low the telomerase activity was markedly reduced, independently of the expression of other important components of the telomerase complex such as telomerase reverse transcriptase (TERT). *In vitro* experiments showed that reduction of dyskerin expression affect telomerase activity through the reduction of TERC. Only when TERC levels were strongly reduced telomerase activity was hindered. Retroviral mediated over-expression of TERC abolished the telomerase impairment due to dyskerin knock down. In conclusion, our results indicated that, beside its effect on ribosome biogenesis, the levels of dyskerin in cancer cells modulate telomerase activity through the regulation of TERC levels, independently of TERT expression. This should be taken into consideration when utilizing TERT expression as a surrogate indicator of telomerase activity in tumour pathology.

Keywords: Breast carcinomas, dyskerin, telomerase, TERC, TERT

1. Introduction

Mutation of the *DKC1* gene causes the rare skin, mucosal and bone marrow failure syndrome termed X-linked dyskeratosis congenita (DC) [1]. The *DKC1* gene product, dyskerin, is a nucleolar protein with various functions. On the one hand it binds to a group of small nucleolar RNAs (snoRNAs) and of small Cajal body-specific RNAs (scaRNAs) containing sequence elements termed H/ACA [2]. These snoRNAs guide the dyskerin-mediated isomerization of specific uridine residues to pseudouridine in ribosomal RNA (rRNA) and small nuclear RNA (snRNA), respectively [2,3]. On the other hand dyskerin binds to the telomerase RNA component (TERC), which also contains

an H/ACA sequence [4]. Through this latter action dyskerin allows TERC stabilization and proper functioning of the telomerase complex. In X-linked DC, failure in this dyskerin function results in the degradation of TERC and consequent impairment of telomerase activity [4,5]. One well established feature of X-linked DC is an increased susceptibility to cancer. Approximately 10% of patients develop malignancies before the age of 30 [1]. Increased cancer susceptibility has also been observed in the *DKC1* hypomorphic mouse, in which dyskerin expression is reduced by means of gene targeting to approximately 30% of normal levels [6,7]. These observations indicate that, at least in DC patients and in *DKC1* hypomorphic mice, dyskerin may behave as a tumour suppressor, prompting cancer when not functioning properly [8].

We recently demonstrated that in primary breast carcinomas dyskerin expression is highly variable and strictly related to its major functions. In particular, in tumours expressing low amounts of dyskerin both

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rRNA pseudouridylation and TERC levels are decreased [9]. Interestingly, in breast carcinomas dyskerin levels also prove to be significantly associated with survival: tumours expressing low dyskerin levels were characterized by a better prognosis [9]. Telomerase activity is known to be associated with prognosis in breast cancer, as well: the higher the activity, the worse the prognosis [10,11]. TERC levels are, in general, very abundant in most neoplastic and non-neoplastic cell types, and its quantity is not considered to be related to telomerase activity [12]. However, if the levels of this essential component of the telomerase complex are strongly reduced, telomerase function may be impaired [13]. Thus in breast carcinomas characterized by very low dyskerin levels telomerase function could be impaired because of a strong reduction in TERC. If demonstrated, this defect in telomerase function might explain the less aggressive clinical behaviour of tumours expressing low dyskerin levels.

In this study we evaluated the relationship between dyskerin expression and telomerase activity in a series of human breast carcinomas. Since the results obtained suggested a threshold level effect of dyskerin on telomerase activity, we also carried out an *in vitro* study in order to verify this hypothesis. For this purpose we evaluated the relationship among dyskerin expression levels, TERC and telomerase activity on the breast cancer derived MCF-7 cell line. Also in this *in vitro* model we found that dyskerin levels, acting on TERC, influence telomerase activity in a threshold-dependent fashion.

2. Materials and methods

2.1. Patients

Sixty-one carcinomas of the breast were studied. Cases were selected, solely on the basis of frozen tissue availability, from a series of consecutive patients who underwent surgical resection for primary infiltrating carcinomas of the breast at the Surgical Department of the University of Bologna in 2006. This non-interventional study was approved by the local ethical committee. Written informed consent was obtained from every patient. Tumours were histologically classified according to the World Health Organisation criteria. Estrogen receptor (ER) and progesterone receptor (PGR) status and Ki67 antigen expression were assessed on histological sections by standard immunohistochemistry. The main clinical and histopathological characteristics [14,15] of the studied population are summarized in Table 1.

2.2. Real time RT-PCR

Total RNA was extracted from frozen samples using Trizol reagent (Invitrogen, Darmstadt, Germany). For each sample, 5 µg of total RNA was reverse transcribed using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. The cDNA was subjected to real-time PCR analysis in a gene Amp 7000 sequence detection system (Applied Biosystems) using the TaqMan approach. Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. For each sample three replicates were analyzed. Sets of primers and fluorogenic probes specific for dyskerin and TERT mRNA were purchased from Applied Biosystems (assay on demand) while TERC specific primer and probe were synthesized as described [16]. The relative amounts of the target genes studied were calculated using the expression of human β -glucuronidase as an endogenous control (Applied Biosystems). The final results, were determined as follows: $N \text{ target} = 2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ calibrator})}$, where ΔC_t values of the sample and calibrator were determined by subtracting the C_t value of the endogenous control gene from the C_t value of each target gene. As a calibrator, in each real time session an aliquot from a single large cDNA preparation from the 1301 cell line was used.

2.3. DKC1 mRNA knockdown using siRNAs

Asynchronously growing MCF-7 cells were subjected to RNA interference (RNAi) for DKC1 mRNA. Cells were grown in standard conditions and transfected with 3 different DKC1 siRNA sequences or with scrambled sequences matched for GC content as a negative control (Stealth RNAi, Invitrogen). Transfections were performed utilizing Lipofectamin 2000 and following the procedures recommended by the manufacturer (Invitrogen).

2.3.1. Evaluation of telomerase activity

Both in tissue samples and in cell lines telomerase activity was measured by a non-radioactive variant of the telomeric repeat amplification protocol (TRAP) [17]. Telomerase activity in tissue samples was measured using the Telomerase PCR ELISA kit (Roche applied sciences, Milan, Italy). For each condition 1 µg of tissue extract was analyzed. After the initial incubation step, the elongation products were amplified by PCR for 29 cycles. To qualitatively control the TRAP prod-

Table 1
Clinical and histopathological characteristics of the studied series

	Whole series <i>n</i> = 61 (%)	Dyskerin expression	<i>p</i>	Telomerase activity	<i>p</i>	TERT expression	<i>p</i>
Age							
<50%	9 (14.7)	0.46 ± 0.28	0.41	0.91 ± 1.27	0.31	92.8 ± 82.4	0.20
≥50%	52 (85.3)	0.53 ± 0.25		0.37 ± 0.67		209.3 ± 298.3	
Histology							
Ductal carcinomas	56 (91.8)	0.53 ± 0.27	0.02	0.44 ± 0.77	0.59	197.7 ± 287.3	0.30
Lobular carcinomas	5 (8.2)	0.27 ± 0.12		0.44 ± 0.87		126.8 ± 177.0	
Tumor size							
pT1	37 (60.7)	0.53 ± 0.22	0.38	0.43 ± 0.76	0.71	195.8 ± 318.2	0.95
pT2	21 (34.4)	0.52 ± 0.34		0.45 ± 0.78		177.4 ± 197.5	
pT3	3 (3.3)	0.31 ± 0.27		0.63 ± 1.09		238.3 ± 322.6	
Histological grade							
G1	5 (8.2)	0.44 ± 0.29	0.53	0.15 ± 0.34	0.20	45.4 ± 48.5	0.06
G2	31 (50.8)	0.50 ± 0.27		0.47 ± 0.86		146.1 ± 137.0	
G3	25 (41)	0.56 ± 0.27		0.48 ± 0.73		281.4 ± 399.4	
N-status							
N0	29 (47.5)	0.53 ± 0.27	0.08	0.51 ± 0.95	0.42	142.0 ± 125.6	0.93
N+	32 (52.5)	0.27 ± 0.12		0.39 ± 0.57		231.5 ± 361.4	
ER-status (LI)							
<10%	15 (24.6)	0.44 ± 0.23	0.13	0.66 ± 0.88	0.83	144.2 ± 101.2	0.75
≥10%	46 (75.4)	0.61 ± 0.28		0.58 ± 0.88		196.0 ± 194.3	
PGR-status (LI)							
<10%	26 (42.6)	0.58 ± 0.34	0.86	0.70 ± 0.75	0.16	226.7 ± 253.9	0.96
≥10%	35 (57.4)	0.57 ± 0.23		0.53 ± 0.96		161.5 ± 114.0	
Ki67-LI							
<20%	38 (62.3)	0.56 ± 0.28	0.55	0.45 ± 0.60	0.36	129.3 ± 102.3	0.03
≥20%	23 (37.7)	0.60 ± 0.28		0.82 ± 1.16		279.2 ± 238.0	

uct (amount, ladder appearance) from primary tumour specimens, the polyacrylamide gel electrophoresis separated PCR products were visualized by chemoluminescence according to the manufacturer's protocol. On the other hand, in cell lines, to obtain quantitatively more precise results when evaluating a small variation in telomerase activity, a modified protocol suitable for real time PCR [15] was applied, using the quantitative telomerase detection kit (Allied Biotech Inc., Ijamsville, MD, USA).

2.4. Western blot analysis

Cells were harvested at different time points in lysis buffer (KH₂PO₄ 0.1 M, pH 7.4, 1% Igepal CA 630; Sigma Chemical, St. Louis, MO) and immediately homogenized. Lysates were then incubated for 10 min on ice and centrifuged for 45 min at 20,000*g*. All steps were performed at 4°C and all solutions contained a cocktail of protease inhibitors (Complete, Roche Diag-

nostics Italia, Milan, Italy). Twenty micrograms of proteins from each sample were electrophoresed in 10% SDS-PAGE. After electro-blotting, filters were developed according to the enhanced chemoluminescence kit (GE healthcare Bioscience Corporation) using an anti-dyskerin rabbit polyclonal antibody diluted 1:400 (Atlas Antibodies AB, AlbaNova University Center, Stockholm, Sweden) or an anti-β-actin mouse monoclonal antibody (Sigma).

2.5. Retroviral mediated TERC overexpression

MCF-7 breast cancer cells were retrovirally infected by standard methods using the pBabe-Puro-TER vector generated by Wong et al. [5]. Chemical selections began 48 h after infection vector with 2 μg/ml puromycin. Viable cells after at least one week of selection cells were used for subsequent evaluations.

2.6. Statistical analysis

Correlations between continuous variables were computed by means of the Spearman rank-correlation coefficient. Associations between continuous and categorical variables were analyzed using the Mann–Whitney test. Statistical analysis was performed using the SPSS statistical software package (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA). Values for p less than 0.05 were regarded as statistically significant.

3. Results

The relationship between dyskerin expression and telomerase activity was evaluated in a series of human primary breast carcinomas. Sixty-one invasive breast carcinomas were studied. In these tumours dyskerin mRNA expression was evaluated by real time RT-PCR. The relative dyskerin expression ranged from 0.04 to 1.53 arbitrary units (mean 0.52 ± 0.28 SD). Dyskerin expression in relationship to the main clinical and histopathological characteristics of the stud-

ied population is reported in Table 1. In a different group of patients we had previously demonstrated that dyskerin levels are strictly related to TERC levels [9]. This association was evaluated in the present study too, using an independent series. The mean relative TERC level, evaluated by real time RT-PCR, was 0.47 arbitrary units (range 0.01–1.71, SD 0.4). Comparison of the TERC values obtained with the dyskerin mRNA expression data confirmed a direct correlation between these two parameters ($r = 0.611$, $p < 0.001$) (Fig. 1(a)). Telomerase activity was evaluated by TRAP assay performed on lysates obtained from frozen tumour specimens (Fig. 1(b)). The mean relative telomerase activity was 0.45 arbitrary units (range 0–3.7, SD 0.77). Telomerase activity in relationship to the main clinical and histopathological characteristics of the studied population is reported in Table 1. Telomerase activity did not prove significantly related to dyskerin expression ($r = 0.062$, $p = 0.637$) or to TERC levels ($r = 0.031$, $p = 0.874$). However, a significant difference was found (Mann–Whitney's $U = 173$, $p = 0.048$) on comparing the telomerase activity of the tumours characterized by a very low dyskerin expression (relative ex-

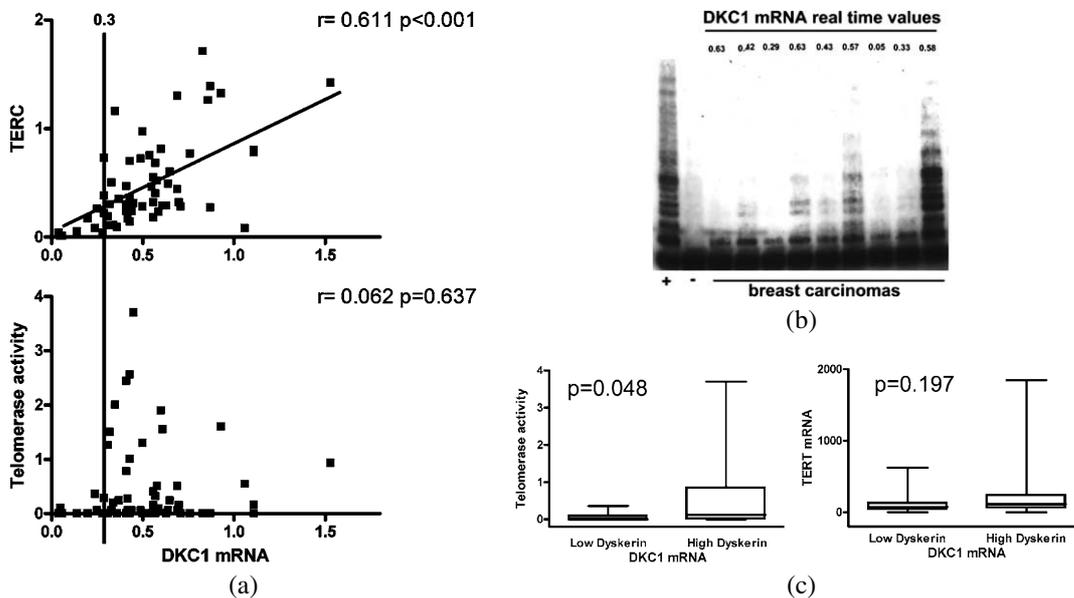


Fig. 1. Relationship between dyskerin expression and TERC, TERT and telomerase activity in breast carcinomas. Dyskerin mRNA levels proved strictly related to TERC levels (a, upper panel). No linear relationship was found when comparing dyskerin mRNA expression and telomerase activity, though a subset of tumours characterized by reduced dyskerin expression (relative expression < 0.3) were characterized by a significant reduction in telomerase activity ($p = 0.048$; (a) lower panel, (b, c) left panel) as compared to tumours with higher dyskerin expression (> 0.3). (b) Evaluation of telomerase activity by TRAP analysis. The relative expression of DKC1 mRNA is also shown. “+” telomerase positive control; “-” heat inactivated telomerase negative control. (c) Box and whiskers graph of telomerase activity and TERT relative expression obtained in samples with low (< 0.3) and high (> 0.3) dyskerin expression. While telomerase activity resulted significantly reduced in tumor expressing low dyskerin levels ($p = 0.048$), no significant difference was found when comparing TERT expression of the two groups ($p = 0.197$).

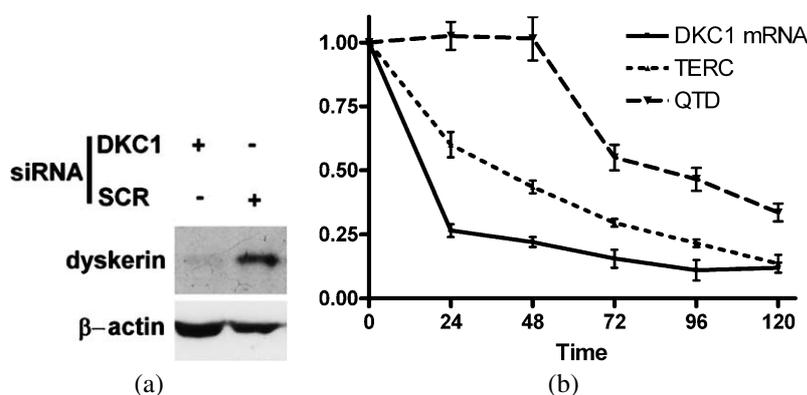


Fig. 2. Effect of DKC1 mRNA reduction by RNAi on TERC levels and telomerase activity. Twenty-four hours after transfection of DKC1 specific siRNAs a steep decrease in dyskerin levels is observed by Western blot analysis in the MCF-7 cell line (a). (b) shows a time course analysis of DKC1 mRNA levels, TERC levels by real time RT-PCR and telomerase activity by TRAP-real time assay (QTD) in the MCF-7 cell line after DKC1 specific RNAi. All measures are expressed as percentages, relative to the values of controls at each time point. Telomerase activity did not change even in the presence of a considerable reduction in TERC levels. When the TERC level was reduced below a certain threshold telomerase activity was impaired. The experiment was repeated in triplicate. Bars: standard deviations.

pression <0.3 , 11 cases) to those with a higher expression (>0.3 , mean telomerase relative value, 50 cases – Fig. 1(a, b)). Being a component of the telomerase enzymatic complex, the levels of dyskerin could be co-regulated according to the expression of other elements in the complex. We then measured the levels of the mRNA coding for telomerase reverse transcriptase (TERT), also known as telomerase catalytic component, which is also used in some studies as a surrogate marker for telomerase activity [10,17]. TERT mRNA mean relative expression was 19.1 (range 0–184.5, SD 27.9). TERT expression in relationship to the main clinical and histopathological characteristics of the studied population is reported in Table 1. No significant relationship was found between dyskerin and TERT mRNA expressions ($r = 0.229$, $p = 0.078$), indicating that dyskerin and TERT mRNA expression are independently regulated. Moreover, the mean TERT relative expression of tumours having low dyskerin expression (<0.3) was not significantly different from those expressing higher dyskerin levels ($p = 0.197$, Fig. 1(c)).

Our results indicate that in breast cancers dyskerin levels are not related to telomerase activity in a linear way. However, if dyskerin is expressed below a given threshold, a reduction in telomerase activity is observed independently of the expression of other important components of the telomerase complex such as TERT.

In order to experimentally evaluate the relationship between dyskerin expression and telomerase activity, we performed DKC1 mRNA knock-down by RNA in-

terference on the breast cancer derived MCF-7 cell line monitoring the levels of DKC1 mRNA, dyskerin, TERC and telomerase activity at 24 hourly intervals. Through this RNAi approach we managed rapidly to degrade 70–90% of DKC1 mRNA after 24 h (see Fig. 2(a)), thus almost completely blocking the *de novo* synthesis of dyskerin. We also monitored dyskerin protein levels by Western blot analysis. Our results showed a steep reduction of dyskerin, starting 24 h after siRNA transfection (Fig. 2(b)). Moreover, TERC levels reduced progressively after dyskerin reduction. Interestingly, telomerase activity remained broadly unchanged even in the presence of a considerable reduction in TERC levels. However, when the relative amount of TERC was reduced below a certain threshold, telomerase activity turned out to be strongly impaired.

In order to define if the reduction of dyskerin expression reduced telomerase activity specifically through the loss of TERC stability we retrovirally transduced MCF-7 cells with a TERC expression vector, thus generating the MCF-7 pBabe TER cells. These cells displayed higher TERC levels in comparison to MCF-7 parental cells and to control MCF-7 cells infected using an empty pBabe retroviral vector (MCF-7 pBabe empty vector, Fig. 3). Due to its intense transcription, the decrease in TERC levels 96 h after DKC1 mRNA specific RNAi in the MCF-7 pBabe TER cells was much less intense than that observed in control MCF-7 pBabe empty vector cells. This persistence of TERC was sufficient to completely revert the dyskerin mediated telomerase impairment (Fig. 3(b)).

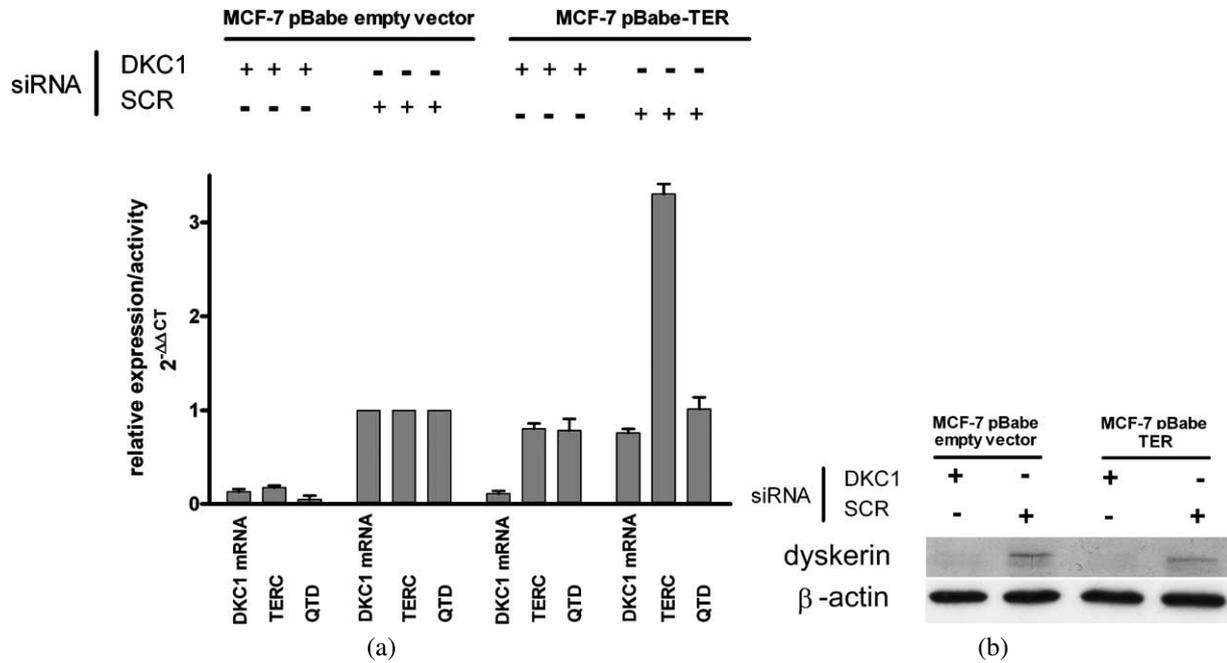


Fig. 3. The telomerase activity impairment after dyskerin reduction is mediated by TERC. MCF-7 cells overexpressing TERC (MCF-7 pBabe-TER) were generated by retroviral mediated transduction. (a) The decrease in TERC levels 96 h after DKC1 mRNA specific RNAi in MCF-7 pBabe-TER is much less intense than that observed in control MCF-7 generated by the infection with an empty pBabe-Puro vector (MCF-7 pBabe empty vector). Even in presence of a steep decrease of DKC1mRNA and dyskerin, the persistence of TERC is sufficient to completely revert the observed telomerase impairment (QTD – quantitative telomerase detection: telomerase activity measured by real time PCR). All measures are expressed as arbitrary values, relative to the expression or the activity of controls (MCF-7 pBabe empty vector cells transfected with control siRNA – SCR). The experiment was repeated in triplicate. Bars: standard deviations. (b) Western blot analysis demonstrating that both in MCF-7 pBabe-TER and in MCF-7 pBabe empty vector DKC1 mRNA knock down resulted in a strong reduction of dyskerin levels after 96 h.

In sum, these *in vitro* results indicate that dyskerin expression could not affect telomerase activity in a direct linear way. However, telomerase impairment is only observed when, as a result of dyskerin reduction, TERC levels are reduced below a critical threshold.

4. Discussion

In this study, the relationship between dyskerin expression and telomerase activity was evaluated in a series of human breast carcinomas. We observed that when dyskerin expression was very low, the telomerase activity was also significantly reduced. The mechanism responsible for this reduction was explained by *in vitro* RNAi experiments on the MCF-7 cancer cell line, indicating that dyskerin levels influence telomerase activity in a threshold-dependent fashion through its action on TERC.

Telomerase activity in cancer cells is highly variable and in most cases directly linked to the expression

of TERT, which is commonly considered the limiting component of the telomerase complex [10,18–21]. Our results indicated that in a subset of breast carcinomas (11 out of 61 – 18%) low levels of telomerase activity were associated with the lack of dyskerin, rather than with the TERT expression, which in these selected cases was actually not reduced. This finding suggests that dyskerin levels may themselves determine telomerase activity.

It has been reported that in acute leukemia, in spite of a high expression of TERT, in some cases telomerase activity was low in association with low TERC levels, raising the possibility that the alteration of the TERC/TERT ratio may affect telomerase activity [22]. This could also occur in breast carcinomas, with the important contribution of dyskerin to regulating TERC levels. In addition we found that tumours with high dyskerin levels were characterized either by an increase or a reduction in telomerase activity, indicating that, similarly to what is described to happen with TERC [23], high dyskerin levels are not sufficient to generate an increase in telomerase activity.

We also evaluated the relationships among dyskerin expression, TERC and telomerase activity in a time course experiment performed on MCF-7 breast carcinoma cell line, in which dyskerin expression had been reduced by RNAi. The DKC1 mRNA and dyskerin levels rapidly decreased 24 h after siRNA transfection. After that, TERC levels were also reduced, reaching equilibrium with dyskerin. In these experimental conditions we found that, after dyskerin knock-down, telomerase activity was only reduced when TERC levels were also reduced below a certain threshold level. This indicated that TERC stabilization by dyskerin was essential for the maintenance of telomerase activity. This was clearly demonstrated by the fact that in MCF-7 cell line the retroviral mediated over-expression of TERC abolished the reduction of telomerase activity after the knock down of dyskerin expression, similarly to what is observed to occur in cells derived from DC patients bearing DKC1 mutations [4]. These results were consistent with observations carried in human breast primary tumours, providing evidence that, in acting on TERC, dyskerin levels directly affect the telomerase activity but only when TERC levels are reduced below a critical threshold.

The present results may also explain our previous observations from an independent series of breast carcinomas in which cases with very low level of dyskerin expression were characterized by good prognosis [9]. Telomerase activity is, indeed, an important prognostic determinant in breast cancer [10,11]. Hence low dyskerin-expressing tumours can be characterized by a better clinical outcome in consequence of a limited telomerase function which impairs their growth capability.

These results also represent a challenge to the methods which quantify telomerase activity through the evaluation of TERT levels. Due to the routine manipulation procedures, the preservation of telomerase activity in surgical tumour tissue specimens can be problematic. In order to bypass this problems it has been proposed to measure the levels of TERT mRNA as a surrogate indicator of telomerase activity. Indeed in different tumour types including breast cancer, TERT expression results directly associated with telomerase activity [21,23,24]. However, the present results indicate that, by means of this approach it is not possible to achieve a correct evaluation of telomerase activity in tumours expressing low dyskerin levels. For these cases a reliable quantification of telomerase activity remains possible only through a TRAP based enzymatic

assay or through the quantification of both TERT mRNA and TERC levels.

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