

Evaluation of dyskerin expression and the Cajal body protein WRAP53 β as potential prognostic markers for patients with primary vaginal carcinoma

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Abstract. Primary vaginal cancer (PVC) is a rare gynaecological malignancy, which, at present, lacks appropriate biomarkers for prognosis. The proteins dyskerin and WD repeat containing antisense to TP53 (WRAP53 β), both of which exert their functions in the telomerase holoenzyme complex, have been shown to be upregulated in different cancer types. These proteins have also been proposed as prognostic markers in some types of cancer. The aim of the present study was to examine the expression patterns of dyskerin and WRAP53 β in patients with PVC. Moreover, as part of a search for effective biomarkers to evaluate prognosis in PVC, the expression of these two proteins and their

potential association with clinical variables and survival were also evaluated. The expression of dyskerin and WRAP53 β was assessed in PVC tumour samples from 68 patients using immunohistochemistry. The majority of tumour samples showed low and moderate expression levels of dyskerin. Upregulation of dyskerin in tumour samples was significantly associated with a shorter survival time and a poorer cancer-specific survival rate. WRAP53 β was also expressed in most of the cells but was not significantly associated with clinical variables or survival. This study demonstrates that upregulation of dyskerin is significantly associated with poor prognosis. Thus, dyskerin may serve as a promising prognostic marker and a potential putative therapeutic target in PVC.

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Abbreviations: PVC, primary vaginal carcinoma; TERT, telomerase reverse transcriptase; TERC, telomerase RNA complex; HPV, human papillomavirus; IHC, immunohistochemical; WRAP53 β , WD repeat containing antisense to TP53

Key words: primary vaginal carcinoma, dyskerin, Cajal body, WRAP53 β , biomarker, prognosis, survival

Introduction

Primary vaginal carcinoma (PVC) accounts for 1-2% of all gynaecological malignancies and mainly affects postmenopausal women, the majority of whom are diagnosed at an early stage (1-3). Radiation therapy, with or without concurrent chemotherapy, is considered to be the treatment of choice and results in 5-year cancer-specific survival rates of ~70% (4). Due to its rarity, few studies have been conducted on PVC, and therefore knowledge concerning biological factors and biomarkers, both diagnostic and prognostic, is limited. Established prognostic factors include age, tumour size, and stage (5-8). Several molecular biomarkers have been proposed, including p16, Ki67, p53, EGFR, VEGF and human papillomavirus (HPV) infection, although findings regarding these markers have been inconsistent (7,9,10). Therefore,

identification of new biomarkers is essential for the improvement of diagnosis, treatment outcome and prognosis for patients with PVC.

Cancer cells are able to proliferate constitutively (11). One important mechanism that supports endless proliferation is the addition of telomere repeats, which protect chromosomes from shortening. This process is carried out by the telomerase enzyme, a ribonucleoprotein complex consisting of two components: The telomerase reverse transcriptase (TERT) and the telomerase RNA complex (TERC) (12). Additional factors required for the catalytical activity of telomerase include dyskerin (also known as dyskerin pseudouridine synthase 1) and the WD repeat containing antisense to TP53 β (WRAP53 β) protein (also known as telomerase Cajal body protein 1 or as WD repeat domain 79) (13). Dyskerin serves as a backbone of the telomerase complex. WRAP53 β is required for telomere synthesis in human cancer cells, as this protein localises telomerase to telomeres (14). Dyskerin and WRAP53 β are usually localised in nuclear organelles known as Cajal bodies, where they are involved in both the biogenesis of telomerase and in spliceosomal machinery (14,15).

Dyskerin, a pseudouridine synthase, is also found in the nucleoli of cells (16), where it is responsible for the modification of ribosomal RNA molecules important to ribosome biogenesis. When dysregulated, this protein has been associated with various cancer types, including breast cancer (17), renal cancer (18) pituitary tumours (19) and glioma (20). In addition, increased expression of dyskerin has often been associated with worse prognosis (16,18,21-23). To our knowledge, dyskerin has not previously been studied in relation to gynaecological malignancies.

WRAP53 β , originally identified as an antisense gene to the TP53 tumour suppressor (24), is a scaffolding protein involved in the intracellular trafficking of RNA, telomerase and DNA repair proteins. WRAP53 β has been linked to a variety of cellular processes, including the maintenance of Cajal bodies (24), telomere elongation (14) and DNA repair (25). Loss of WRAP53 β is associated with poor prognosis in head and neck (26), breast (27) and ovarian cancer (28), suggesting a tumour suppressor role (29,30). Although upregulation of WRAP53 β has also been reported in cancer, correlation with patient survival has been inconsistent (31-33). Instead, this upregulation, which is known to occur in precancerous lesions, may reflect WRAP53 β involvement in DNA repair in order to constrain tumour progression (29,30).

The present study aimed to examine the expression patterns of dyskerin and WRAP53 β in patients with PVC. Moreover, as part of a search for effective biomarkers to evaluate prognosis in PVC, the expression of these two proteins and their potential association with clinical variables and survival was also evaluated.

Materials and methods

Sample collection. The present study is based on archived diagnostic PVC tumour samples from a consecutive cohort of 81 women. The inclusion criteria were women diagnosed with and treated for PVC between January 1975 and December 2002 at Örebro University Hospital or at the central hospitals in Eskilstuna, Västerås, and Karlstad (9). Seven cases

were excluded after immunohistochemical evaluation due to insufficient tumour samples.

The clinical characteristics of this cohort have been previously described in a study by Larsson *et al* (9), including information on age at diagnosis (mean, 69.4 years; range, 37-90 years), tumour size, International Federation of Gynecology and Obstetrics (FIGO) stage, tumour localisation, histological type (including basaloid squamous cell carcinoma, non-keratinising squamous cell carcinoma, keratinising squamous cell carcinoma, verrucous squamous cell carcinoma, adenocarcinoma, sarcoma and melanoma, based on World Health Organisation criteria) and tumour grade (34). Treatment and follow-up data for each patient were obtained through hospital records. All patient records were subjected to retrospective follow-up from the time of diagnosis. Median follow-up time for patients who were alive at the end of the study was 121 months (range, 44-290 months). As in the previous protocol by Larsson *et al* (9), complete remission was defined as disappearance of all clinical evidence of disease after primary treatment, while tumour recurrence was defined as detection of cancer after a period of at least 6 months following initial complete remission.

Information on HPV status was also reported in Larsson *et al* (9). Of the 81 tumour samples, 37 were HPV-positive, 34 were HPV-negative, and 10 had insufficient material for HPV detection. Of the 37 HPV-positive cases, 26 (70%) were HPV16-positive, while the remaining 11 were positive for other high-risk HPV genotypes.

Immunohistochemical staining and analysis of dyskerin and WRAP53 β . In all, 68 of the 81 tumour samples were found to be appropriate for immunohistochemical staining and analysis of dyskerin and WRAP53 β . The paraffin-embedded tumour samples were cut into 5- μ m sections and established to be representative by two pathologists (MGK and OG). Paraffin was then dissolved in xylene, and the tissue samples were rehydrated by stepwise washing with 96 and 70% ethanol in phosphate-buffered saline. The tissue samples were then immersed in a 2% solution of H₂O₂ in methanol at room temperature for 30 min to reduce background staining. Epitopes were retrieved by heating in citrate buffer (water bath, 96°C for 15 min), and the tumour samples were then cooled to room temperature. The primary anti-dyskerin (cat. no. sc-373956; Santa Cruz Biotechnology, Inc.) and anti-WRAP53 (cat. no. PA-2020-100; Innovagen AB) antibodies, diluted 1:200 in a blocking buffer (2% bovine serum albumin, 0.2% Tween-20, 10% glycerol and 0.05% NaN₃ in phosphate-buffered saline; all from Sigma-Aldrich; Merck KGaA) were applied and left to stand for 60 min at room temperature. Protein signals were visualised using the secondary antibodies provided in the EnVision™ Detection Peroxidase/DAB system kit (Dako; Agilent Technologies, Inc.), which was used according to the manufacturer's protocol. Nuclei were stained with Mayer's haematoxylin (Dako; Agilent Technologies, Inc.).

The dyskerin nuclear signal was assessed as: i) Negative (no positive cells observed); ii) weak (1+); iii) moderate (2+); and iv) and strong (3+) by an experienced pathologist (OG), using a light microscope Leica (magnification, x400). Tumour cells were only analysed in sections in which the total number

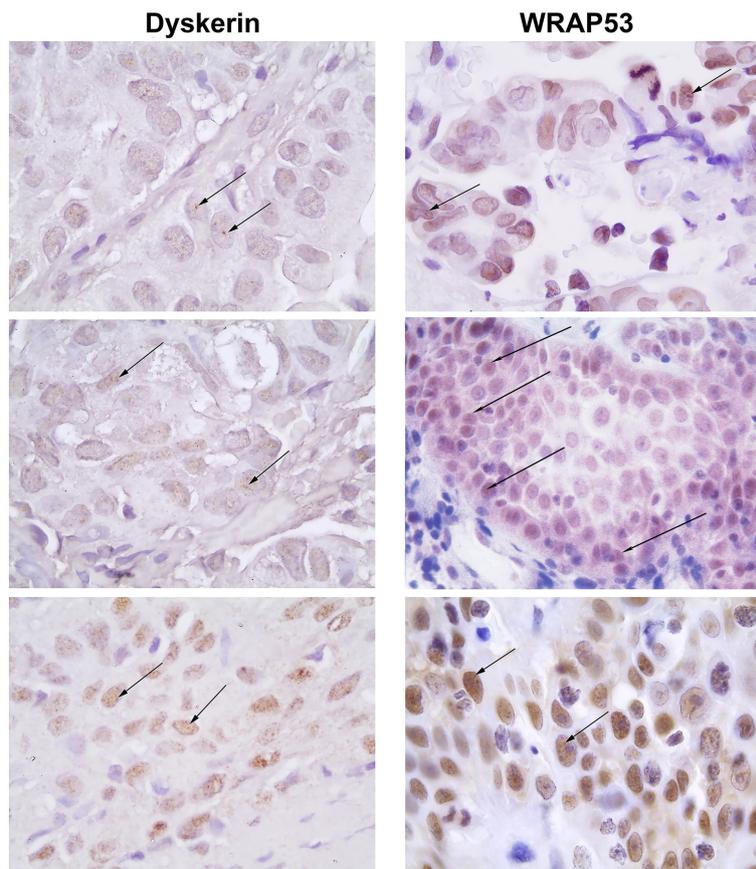


Figure 1. Expression pattern of dyskerin and WRAP53 β in tumour samples of three representative patients with primary vaginal cancer (magnification, x400). The expression of dyskerin and WRAP53 β was assessed using immunohistochemistry (in brown). On the left column, dyskerin is indicated with arrows and located in the nucleoli (top panel), nuclear bodies (middle panel) and is dispersed in nucleoplasm (bottom panel). On the right column, arrows indicate presence of WRAP53 β in nuclear bodies located in the nucleoplasm, i.e. not nucleoli. Nuclei are shown in blue. WRAP53 β , WD repeat containing antisense to TP53 β .

of cells was ≥ 400 (minimum of 5 fields). The location of the protein signals detected within the nucleoplasm and/or in nuclear bodies was also recorded. In the present study, nuclear bodies refer to nucleoplasmic bodies, excluding nucleoli.

Two experienced pathologists (OG and MGK) carried out microscopic evaluation of WRAP53 β signals based on the fraction of stained tumour cells and on staining intensity. The percentage of positive cells was categorised into four, semi-quantitative groups: i) 0, negative; ii) 1, <25%; iii) 2, 25-50%; and iv) 3, >50% of cells (MKG). In addition, staining intensity was graded as: i) 0, negative (no positive cells observed); ii) 1, weak; iii) 2, moderate; and iv) 3, strong.

Statistical analysis. Pearson's χ^2 test or Fisher's exact tests were used to analyse the association between ordinal variables and IHC parameters. An independent-sample t-test was used to analyse the differences between the means of the groups. Survival analysis according to the expression of dyskerin and WRAP53 β is presented as a Kaplan-Meier graph, and a log-rank test was used to compare the different dichotomised groups. Multivariate analysis of different prognostic factors, such as age at diagnosis, tumour size, histology and FIGO stage, was performed using the Cox proportional-hazards model for survival outcome. Statistical analysis was carried out using SPSS 19 software (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. IHC analysis of dyskerin staining intensity and localisation pattern in tumour samples of patients with primary vaginal cancer.

IHC parameter	n (%)
Dyskerin IHC staining intensity	
Negative (no positive cells)	1 (1.5)
1+ (weak)	54 (79.4)
2+ (moderate)	5 (7.4)
3+ (strong)	8 (11.7)
Dyskerin localisation	
Nuclear bodies (negative nucleoplasm)	9 (13.2)
Nucleoplasm (negative nuclear bodies)	15 (22.1)
Nuclear bodies + nucleoplasm	39 (57.3)
No staining or very weak	5 (7.3)
IHC, immunohistochemical.	

Results

Immunohistochemical analysis of dyskerin. Immunohistochemical analysis of 68 tumour samples revealed varying degrees of nuclear expression of dyskerin (Fig. 1 and Table I).

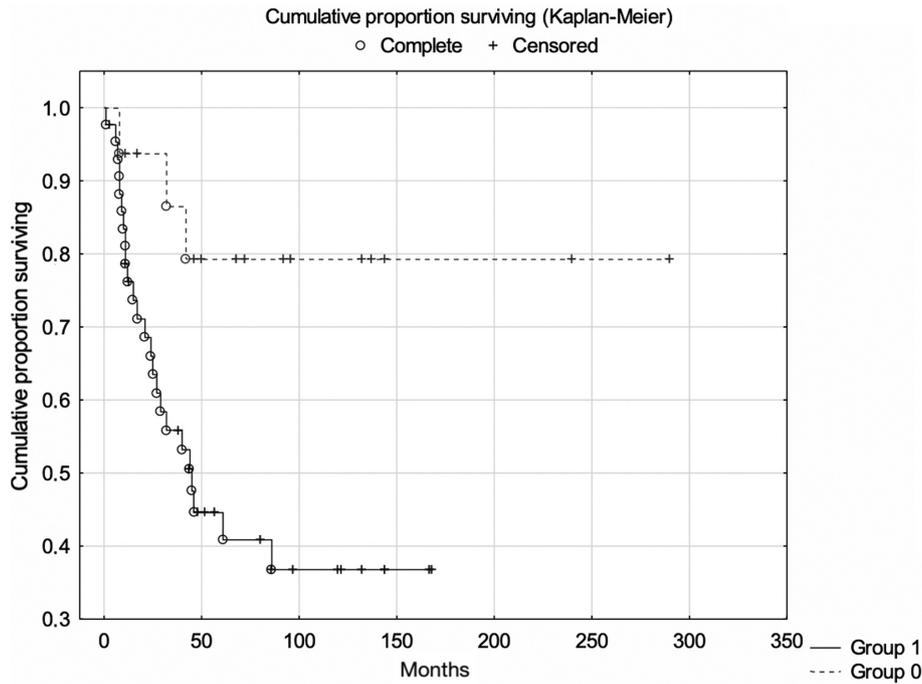


Figure 2. Cancer-specific survival rate vs. intensity of dyskerin staining. Group 0, negative or 1+ staining; group 1, 2+ or 3+ staining. The difference in survival rate was highly significant between the two groups (log-rank test; $P=0.009$).

The majority of cells (79%) demonstrated a weak staining intensity of dyskerin, whereas 7% showed an moderate and 11% a strong staining intensity. Most of the staining was seen in nuclear bodies + nucleoplasm (57%), not only in nucleoli.

Examination of dyskerin staining intensity in relation to clinical variables (Table II) revealed no association between the expression of dyskerin and FIGO stage at diagnosis (Pearson's χ^2 ; $P=0.509$), nor with tumour localisation within the vagina ($P=0.644$). Although a high expression of dyskerin was more frequently observed in HPV-negative (81%) compared with in HPV-positive tumour samples (66%), this difference was not statistically significant ($P=0.20$) (data not shown). Examination of the relationship between the expression of dyskerin and histological type revealed that high expression was more frequently observed in basaloid and keratinising tumours (87%) than in other histological types (59%) ($P=0.015$; data not shown). High expression of dyskerin was also significantly associated with poorly differentiated tumours ($P=0.032$) (Table II).

The primary cure rate was lower in patients with high expression of dyskerin in their tumour samples and those with low expression (81% vs. 94%; $P=0.241$); however, this change was not statistically significant. Similarly, overall recurrence among patients with high expression of dyskerin was 40%, compared to 19% among those with low expression ($P=0.134$). Moreover, 13/14 recurrent tumours with distant metastasis demonstrated a high expression of dyskerin ($P=0.001$) (data not shown).

Examination of dyskerin staining intensity in relation to survival (Table II) revealed significant associations with disease progression and survival. Survival time was significantly shorter in patients with high expression of dyskerin compared with those with low expression (50 months vs.

92 months; $P=0.017$). In addition, 55.8% of patients with high expression of dyskerin had already died from PVC by the time this study was undertaken, while the corresponding figure for patients with low expression was 18.8% ($P=0.039$).

High expression of dyskerin was associated with significantly lower 5-year cancer-specific survival rates (log-rank test; $P=0.009$; Fig. 2). Expression of dyskerin remained a significant and independent prognostic factor after correction for age at diagnosis, tumour size, histological type and FIGO stage (Cox multivariate proportional regression analysis; $P=0.035$; Table III).

Immunohistochemical analysis of WRAP53 β . As with dyskerin, WRAP53 β was expressed in both nuclear bodies (12%; likely Cajal bodies) and in nucleoplasm (16%) (Fig. 3; Table IV). The majority of tumour samples showed expression of WRAP53 β , but no significant association was observed between the percentage of stained cells or staining intensity of WRAP53 β and clinical variables or survival rates (data not shown).

Discussion

The present study demonstrates for the first time to the best of our knowledge that high expression of dyskerin, a protein involved in the modification of nuclear RNA and telomere elongation, is significantly associated with lower cancer-specific survival, as well as with lower overall survival in patients with PVC. One explanation is that dyskerin upregulation may lead to an increase in telomerase supramolecular complex formation, thus increasing the overall catalytic activity of telomerase (Fig. 4). Moreover, the higher dyskerin expression was significantly associated with poorly differentiated tumours. It was

Table II. Correlation analysis of dyskerin expression and clinical variables and survival.

Parameter	Group 0	Group 1	P-value
Mean age at diagnosis, years ^a	70	69	0.878
Mean tumour size, mm	25	23	0.465
FIGO stage, n (%)			0.509
I	7 (43.8)	12 (28.0)	
II	6 (37.5)	19 (44.2)	
III	2 (12.5)	4 (9.3)	
IV	1 (6.3)	8 (18.6)	
Tumour localisation, n (%)			0.644
Upper	5 (31.2)	12 (27.9)	
Middle	4 (25.0)	7 (16.3)	
Lower	4 (25.0)	14 (32.6)	
Entire vagina	2 (12.5)	9 (20.9)	
Middle + lower	0 (0)	1 (2.3)	
Urethra	1 (6.25)	0 (0)	
Histological type, n (%)			0.226
Basaloid	4 (25)	18 (42)	
Non-keratinizing	9 (56)	13 (30.2)	
Keratinizing	0 (0)	8 (18.6)	
Verrucous	1 (6.2)	1 (2.3)	
Adenocarcinoma	2 (12.5)	3 (7)	
Tumour grade, n (%)			0.032
Grade 1	2 (12.5)	6 (14.3)	
Grade 2	7 (43.8)	17 (40.3)	
Grade 3	3 (18.8)	18 (42.9)	
Unknown	4 (25)	1 (2.4)	
HPV status, n (%)			0.360
Negative	5 (31.2)	21 (48.8)	
Positive	11 (69)	21 (48.8)	
Unknown	0 (0)	1 (2.3)	
Primary cure rate, n (%)			0.241
Yes	15 (93.8)	35 (81.4)	
No	1 (6.3)	8 (18.6)	
Recurrence, n (%)			0.134
Yes	3 (18.9)	17 (39.5)	
No	13 (81.3)	26 (60.5)	
Mean time to recurrence, months	29	19	0.511
Localisation of recurrent disease (n=20), n (%)			0.619
Local	2 (66.7)	3 (17.6)	
Regional	0 (0)	1 (5.9)	
Distant metastasis	1 (33.3)	9 (52.9)	
Local + regional + distant metastasis	0 (0)	3 (17.6)	
Regional + distant metastasis	0 (0)	1 (0.6)	
Survival status, n (%)			0.039
Alive	5 (31.3)	8 (18.6)	
Death from disease	3 (18.8)	24 (55.8)	
Death from intercurrent disease	8 (50.0)	11 (25.6)	
Mean survival time, months	50	92	0.017

^aRange, 37-90 years. Group 1, negative or 1+ staining; group 2, 2+ or 3+ staining. n=59 unless otherwise indicated. FIGO, International Federation of Gynecology and Obstetrics; HPV, human papillomavirus.

Table III. Prognostic factors vs. cancer-specific survival rate.

Factor	HR (95% CI)	P-value
FIGO stage (III-IV vs. I-II)	1.669 (0.659-4.224)	ns
Age at diagnosis (per year)	1.020 (0.980-1.062)	ns
Tumour size (per cm)	1.099 (0.738-1.636)	ns
Histology (squamous cell carcinoma vs adenocarcinoma)	1.742 (0.391-7.757)	ns
Dyskerin staining intensity (2-3 vs. 0-1)	3.701 (1.094-12.517)	0.035

Cox multivariate proportional hazards analysis. HR, hazard ratio; CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; ns, not significant.

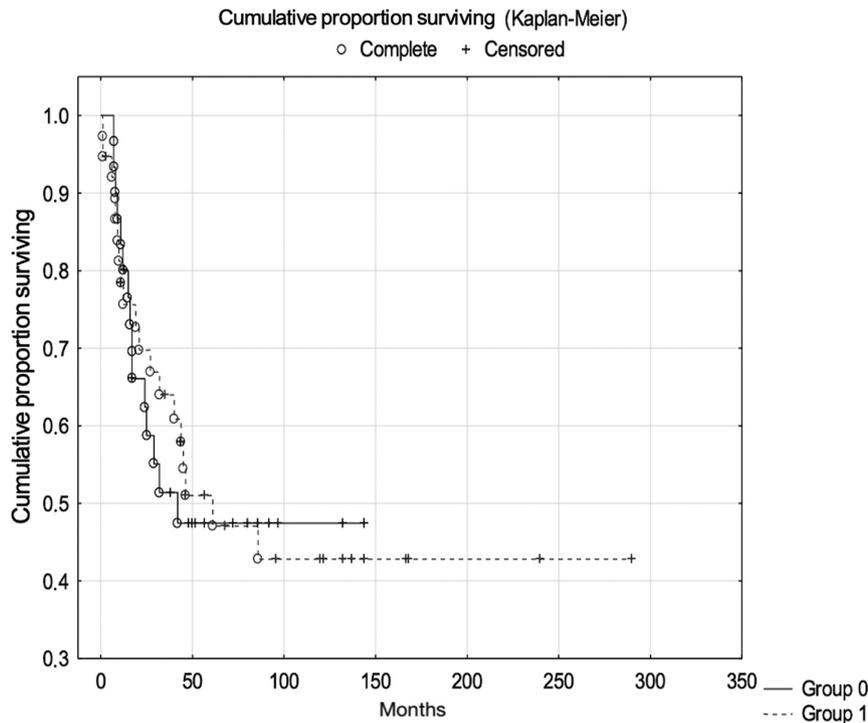


Figure 3. Cancer-specific survival rate vs. intensity of WRAP53 β staining. Group 0, negative or 1+ staining; group 1, 2+ or 3+ staining. There was no significant difference between the two groups (log-rank test; P=0.921). WRAP53 β , WD repeat containing antisense to TP53 β .

also associated with an increased risk of recurrent disease in terms of distant metastasis (Table II). In agreement with these findings, elevated dyskerin levels have been linked to progression and aggressiveness in several tumour types (17,19,20,35) as well as to worse clinical outcomes in breast cancer (16), lung cancer (22), hepatocellular carcinoma (21), renal cell cancer (18) and neuroblastoma (23). Taken together, these findings suggest that dyskerin may be a useful prognostic marker for several types of cancer, including PVC.

A potential limitation of these findings is the limited number of patients included in this retrospective study. This reflects the low incidence of PVC (1) as well as the small population size in Sweden. However, given the non-parametric statistical model used in this study, the assumed risk of an underpowered study design is outweighed by the importance of performing exploratory studies on PVC. Indeed, previous knowledge on the biological factors of PVC has been based on similar sample sizes (2). In addition, due to the rarity of PVC, the present study

was retrospective and based on archived, paraffin-embedded tumour samples instead of fresh-frozen material; the latter would have enabled complementary analyses. Nevertheless, future studies on a larger cohort with additional medical centres to confirm the present findings and to analyse the relationship between the expression of dyskerin and HPV infection in PVC, as well as in other gynaecological malignancies.

The mechanism by which dysregulation of dyskerin contributes to cancer development is debated, although it appears to be linked to both enhanced telomerase activity and protein biogenesis (17,20). Non-small cell lung cancer provides one example of how high dyskerin expression is significantly associated with worse overall survival due to TERC stabilisation. Such stabilisation has been traced to the overexpression of dyskerin rather than to TERC gene amplification (22), which is in line with the idea that dyskerin can modify telomerase activity through the regulation of TERC levels, and independent of TERT expression (36). In the case of prostate cancer,

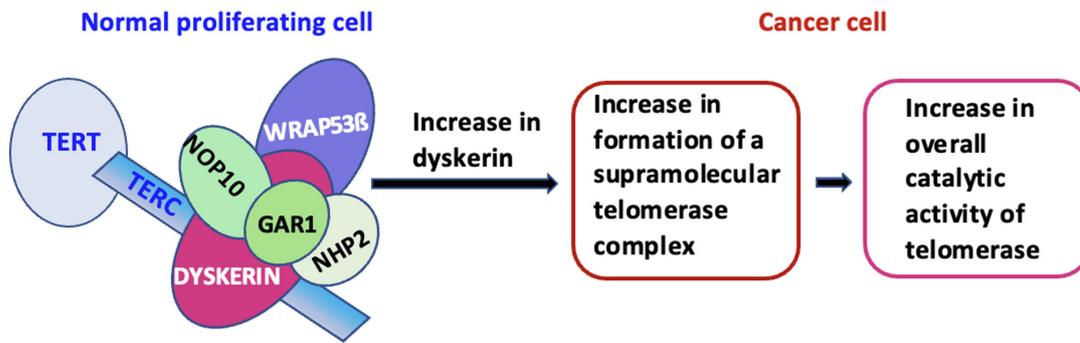


Figure 4. Schematic presentation of a putative role of dyskerin in primary vaginal cancer development. Upregulation of dyskerin leads to increase in telomerase supramolecular complex formation, thereby increasing the overall catalytic activity of telomerase. Dyskerin serves a structural scaffold of the telomerase complex. WRAP53 β , WD repeat containing antisense to TP53 β ; TERT, telomerase reverse transcriptase; TERC, telomerase RNA complex.

Table IV. IHC analysis of WRAP53 β staining intensity, fraction of stained cells and localisation pattern in tumour samples of patients with primary vaginal cancer.

IHC paramter	n (%)
WRAP53 β IHC staining intensity	
0	8 (8.5)
1	22 (32.4)
2	20 (29.4)
3	18 (26.5)
WRAP53 β IHC fraction of stained cells	
0	13 (16.0)
1	32 (39.5)
2	20 (24.7)
3	3 (3.7)
WRAP53 β localisation ^a	
Nuclear bodies	8 (13.1)
Nucleoplasm	11 (18.0)
Nuclear bodies + nucleoplasm	40 (65.6)
Diffuse	2 (3.3)

^aSixty-one samples analyzed; 7 samples excluded as there was no staining in the tumour cells. IHC, immunohistochemical; WRAP53 β , WD repeat containing antisense to TP53 β .

high expression of dyskerin mRNA is associated with more advanced clinical stage and recurrent disease. In this example, dysregulation of dyskerin is associated with enhanced protein biosynthesis rather than with telomerase activity (35). Indeed, loss of dyskerin function has been shown to reduce the amount of pseudouridinylated ribosomal RNA and thereby impair ribosome function and synthesis of proteins (16,37).

Conversely, inactivating mutations of dyskerin cause dyskeratosis congenita, a rare genetic disease associated with a predisposition to cancer development (mainly haematological malignancies and head and neck cancer) (38). Similarly, dyskerin has also been shown to be downregulated in sporadic chronic lymphocytic leukaemia (39).

In light of the current study, upregulation of dyskerin might be associated with the poor prognosis of PVC due to

enhancement of telomerase activity and/or altered protein synthesis. In an attempt to investigate the former, the expression of the WRAP53 β protein, which is known to have a role in transporting dyskerin and the telomerase complex to telomeres as required for telomere elongation (14), was examined. The findings suggested that WRAP53 β was expressed to varying degrees in the majority of PVC tumour samples, but this expression had no significant association with clinical parameters or patient survival. In PVC, the sub-cellular localisation of WRAP53 β could not be linked to survival, in contrast with findings for both breast (27) and head and neck cancer (26). It can be concluded that the role of WRAP53 β as a telomere transporter appears to be intact in PVC and that dyskerin upregulation could therefore result in enhanced telomere elongation. Upregulation of WRAP53 β may also indicate its involvement in the DNA damage response, as suggested by Bergstrand *et al* (29).

Notably, the telomerase complex plays a crucial role in an important oncogenic pathway that stimulates the development and progression of HPV-associated cancer, which relates to the E6 oncogene of HPV that regulates TERT activity (40). Our finding of low expression of dyskerin in HPV-positive PVC tumour samples may suggest that dyskerin is indispensable to telomerase activity in these samples, since the E6 protein activates transcription of the human (h)TERT component of the telomerase complex (41). The association between gene expression linked to hTERT activity, including dyskerin, and malignant progression of HPV-induced cervical lesions, has been previously studied (42), although no correlation was observed between dyskerin expression at the protein level and the severity of precursor lesions. In the present study, dyskerin was detectable in nuclear bodies and/or in nucleoplasm, but not in the cytoplasm. Previous studies have detected dyskerin mainly in the nucleus, as well as in the cytoplasm of cervical precursor lesions (42), renal cell carcinoma (18) and hepatocellular carcinoma cells (21). One possible explanation for this discrepancy with our results may be the use of different dyskerin antibodies.

In summary, the present study demonstrates that upregulation of dyskerin is significantly associated with poor prognosis in PVC. This may be explained by the fact that a high expression of dyskerin may lead to increased telomerase supramolecular complex formation, thereby increasing the overall catalytic activity of telomerase. The future studies on PVC cell lines *in vitro*, using overexpression and downregulation of dyskerin with RNA sequencing analysis may clarify the functional

consequences of dyskerin overexpression. In conclusion, the present findings point to dyskerin as a promising prognostic marker and as a potential putative therapeutic target in PVC.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CR, SA, MF, KH and GLL contributed to study design. GLL, LK and EK performed the immunohistochemical preparation. LK and MGK performed immunohistochemical analysis. BS and CR performed statistical analysis and interpretation of data was performed by CR, BS, and SA. DL helped with the writing of the manuscript and was involved in the planning of the study, formulating the hypothesis and study design. CR drafted the manuscript. BS and CR confirm the authenticity of all the raw data. All authors critically reviewed the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Regional Ethical Review Board in Uppsala, Sweden (approval no. 2008/294), who did not request specific informed consent from patients. Patients were originally orally informed about the clinical research database. After 2003, they were also informed about tissue biobanking in accordance with the Swedish Biobank Act 2002:297. The study was performed in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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