Original Article

Comprehensive Analysis of PTEN in Primary Cutaneous Melanoma

(melanoma / mutation / deletion / loss of expression / PTEN)

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Abstract. Phosphatase and tensin homologue (*PTEN*) is a tumour suppressor gene implicated in tumorigenesis of melanoma, with distinct cytoplasmic and nuclear functions. Cytoplasmic PTEN negatively regulates the PI3K/AKT/mTOR signalling pathway, while nuclear PTEN works as a tumour suppressor. Clinical data suggest that the loss of PTEN function in melanoma is associated with aggressive tumour behaviour. We performed a comprehensive analysis of PTEN in 112 primary cutaneous melanomas including immunohistochemical (IHC), fluorescent *in situ* hybridization (FISH), next-generation sequencing (NGS), and epigenetic analysis. The goal of our study was to: (a) correlate PTEN expression with selected clinico-pathological variables, and assess its prognostic significance; (b) correlate molecular aberrations with PTEN expression to consider the utility of immunohistochemical analysis of PTEN protein expression for screening *PTEN* genetic alterations; (c) review the literature and evaluate the PTEN expression level in melanoma with respect to possible therapeutic targeting. Our results showed that *PTEN* molecular alterations were present in 4/20 (20 %) cases with a loss of expression, 3/11 (27 %) cases with clonal-like expression, and 1/81 (1 %) cases with positive PTEN expression. No *PTEN* promoter methylation was found in any of the cases. Even though the value of our observation is limited by the low number of cases fully evaluated by IHC (112 cases), FISH (19 cases) and NGS (30 cases), our data suggest that IHC is not an appropriate method for the screening of *PTEN* genetic alterations. Our survival analysis suggests that patients with positive cytoplasmic PTEN expression show better disease-free survival ($P < 0.05$).

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Abbreviations: AKT – protein kinase B, ALM – acral lentiginous melanoma, DFS – disease-free survival, FFPE – formalin-fixed paraffin-embedded, FISH – fluorescence *in situ* hybridization, IHC – immunohistochemical, LFS – local recurrence-free survival, MAPK – mitogen-activated protein kinase, MFS – distant metastasis-free survival, NGS – next-generation sequencing (massive parallel sequencing), NM – nodular melanoma, OS – overall survival, PI3K – phosphatidylinositol-3-kinase, PIP2 – phosphatidylinositol-bisphosphate, PIP3 – phosphatidylinositol-3,4,5-triphosphate, *PTEN* – phosphatase and tensin homologue gene, SSM – superficial spreading melanoma, TCGA – The Cancer Genome Atlas, TIL – tumour-infiltrating lymphocytes.

Introduction

Phosphatase and tensin homologue (*PTEN*; OMIM #601728) is a tumour suppressor gene located on chromosome 10q23.31. It is implicated in the carcinogenesis of a large number of tumours including melanoma (Li and Sun, 1998; Poetsch et al., 2001; Song et al., 2012; Milella et al., 2015; Troyer et al., 2015). *PTEN* encodes a multifunctional protein, which acts as a lipid and protein phosphatase, and also acts through other non-enzymatic mechanisms (Milella et al., 2015).

The main function of PTEN lies in negative regulation of the anti-apoptotic PI3K/AKT signalling pathway involved in cancer cell growth, survival, angiogenesis, and metabolism. The lipid phosphatase encoded by *PTEN*

degrades the products of *PI3K* by dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2) (Song et al., 2012). Thus, PTEN is a negative regulator of PI3K, and loss of function of PTEN leads to PIP3 accumulation within the cells and subsequent activation of the downstream AKT signalling (Conde-Perez and Larue, 2012).

PTEN can also function as a tumour suppressor in a PI3K-independent manner by its protein phosphatase activity, which negatively regulates the MAPK pathway and which is also responsible for inhibition of migration and stem cell self-renewal and for inducing cell cycle arrest (Milella et al., 2015). Additionally, PTEN is implicated in the regulation of chromosome stability, DNA repair, and apoptosis through a non-enzymatic mechanism (Dillon and Miller, 2014; Milella et al., 2015).

Germline PTEN mutations are associated with a rare PTEN hamartoma tumour syndrome, which includes Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome, resulting in increased susceptibility to assorted tumours (Song et al., 2012).

Various somatic alterations of the *PTEN* gene are found in 5–20 % of primary melanomas and in about 30–50 % of melanoma cell lines (Deichmann et al., 2002; Conde-Perez and Larue, 2012; Abbotts et al., 2014; Reddy et al., 2017). Some studies have shown that a loss or decrease in PTEN expression in melanoma is associated with aggressive tumour behaviour (Mikhail et al., 2005; Bucheit et al., 2014). The most common inactivating somatic alterations are missense and frameshift mutations, loss of heterozygosity, chromosomal rearrangement, and deletion of *PTEN* (Guldberg et al., 1997; Leonardi et al., 2018). The loss of PTEN function can also result from epigenetic and transcriptional silencing, aberrant protein localization, and post-translational modifications (Dillon and Miller, 2014; Milella et al., 2015).

The *PTEN* mutations frequently coexist with activating *BRAF* mutation, the most common mutation detected in cutaneous melanoma patients (Aguissa-Toure and Li, 2012; Bucheit et al., 2014). Melanomas with concurrent loss of PTEN and activating *BRAF* mutations found together show activated PI3K and MAPK pathways, and they are associated with a worse outcome in melanoma patients (Bucheit et al., 2014; Leonardi et al., 2018). From a clinical point of view, the loss of function is one of the mechanisms responsible for the acquired resistance of *BRAF*-mutated melanoma treated with BRAF inhibitors (Shi et al., 2014; Leonardi et al., 2018). Several interaction partners of the PTEN protein have been described to date. Most importantly, PTEN is a negative regulator of p53, another major tumour suppressor, which regulates cell proliferation and cell death (Freeman et al., 2003; Dillon and Miller, 2014).

In our study we focused on PTEN protein expression and genetic and epigenetic alterations in primary cutaneous melanoma with respect to (a) immunohistochemical analysis of PTEN, in order to correlate PTEN expression with clinico-pathological variables, and to analyse the prognostic significance of PTEN expression, (b) the correlation of genetic, cytogenetic, and epigenetic aberrations with PTEN expression, to consider the utility of immunohistochemical analysis of PTEN protein expression for screening for PTEN genetic alterations, and (c) a review of the literature and evaluation of the PTEN expression level in melanoma with respect to possible therapeutic targeting.

Material and Methods

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were obtained from the archive files of the Institute of Pathology and from the Department of Dermatology and Venereology, First Faculty of Medicine, Charles University and General University Hospital in Prague. A review of the haematoxylin and eosin-stained slides was performed in all cases. In total, 112 FFPE primary cutaneous melanomas were selected for immunohistochemical, epigenetic and mutation analysis (Table 1). The mean age of patients was 61.9 years (median 65.5; range 24 to 93 years). Because of the retrospective character of our study, we used the pT classification from the patients charts (7th edition of TNM Classification of Malignant Tumours) as follows: pT1 (pT1 (≤ 1 mm), pT2 (> 1–2 mm), pT3 (> 2–4 mm), pT4 (> 4 mm) (Sobin et al., 2010). In compliance with the Helsinki Declaration, the study was approved by the Ethics Committee of the General University Hospital in Prague.

Table 1. Characteristics of the 112 patients with primary cutaneous melanoma

Characteristic	N
Gender	
Male	66
Female	46
Age	
≤ 65	56
> 65	56
Tumour stage	
pT1 (≤ 1 mm)	5
pT2 (> 1-2 mm)	24
pt3 (> 2-4 mm)	47
pT4 (> 4 mm)	36
Location	
Head	10
Trunk	67
Upper extremities	17
Lower extremities	18
Histological subtype*	
SSM	67
NM	44
Ulceration	
Yes	52
No	60
Sentinel node positivity	
Yes	19
No	52
NA	41

* one case of ALM was excluded

Immunohistochemical analysis

Immunohistochemical analysis was performed in 4 µm sections of FFPE tissue manually using the avidin-biotin complex method with antibody against PTEN (clone 6H2.1, 1 : 200; Dako, Glostrup, Denmark) in all samples. Antigen retrieval was performed, including pre-treatment in 0.01 M citrate buffer (pH 9.0) for 40 min in a water bath at 98 °C. The expression of PTEN was double-blindly evaluated by two pathologists. The ambiguous cases were evaluated by a third, independent pathologist. Negativity of PTEN was defined as less than 10 % of tumour cells with PTEN expression when compared to internal positive controls (endothelial cells) (Bucheit et al., 2014; Peng et al., 2016). We used the scoring system suggested by Zhou et al. (2000), based on the comparison of tumour cell staining versus staining of adjacent endothelial cells that were present in all the evaluated samples. The cases with staining intensity stronger than the intensity of endothelial cells were scored as 3+, cases with equivalent staining intensity to endothelial cells were scored 2+, cases with intensity weaker than endothelial cells were scored 1+, and absence of staining was scored as 0. There were also 11 cases with a heterogeneous cytoplasmic staining pattern, the so-called clonal-like PTEN expression, which is defined as cases with positive cytoplasmic PTEN expression, showing distinct areas of varying size with a loss of PTEN expression, observed in up to 25 % of the inspected area (Zhou et al., 2000; Goel et al., 2006; Peng et al., 2016). The cytoplasmic IHC staining was categorized as follows: group 0 = negative (scores 3+ and 2+ < 10 %), group 1 = positive (scores 3+ and 2+ ≥ 10 %), group 2 = positive with negative clones. The nuclear IHC staining was categorized as follows: group 0 = negative (< 10 %), group 1 = positive (≥ 10 %). The representative examples of PTEN staining are shown in Fig. 1 (a, b, c).

Fluorescence in situ hybridization

Deletions of the *PTEN* gene (22q12) were analysed using the fluorescent *in situ* hybridization (FISH) method with ZytoLight SPEC PTEN/CEN 10 Dual Color Probe (#Z-2078; ZytoVision, Bremerhaven, Germany). Formalin-fixed paraffin-embedded tissues were sectioned at a thickness of 3 µm and processed according to the manufacturer's instructions. In total, FISH analysis was performed in 31 selected cases comprising 20 cases with negative cytoplasmic, 10 cases with clonal-like, and one case with positive cytoplasmic PTEN expression and detected *PTEN* mutation. Due to the poor quality of samples (especially in samples older than 10 years),

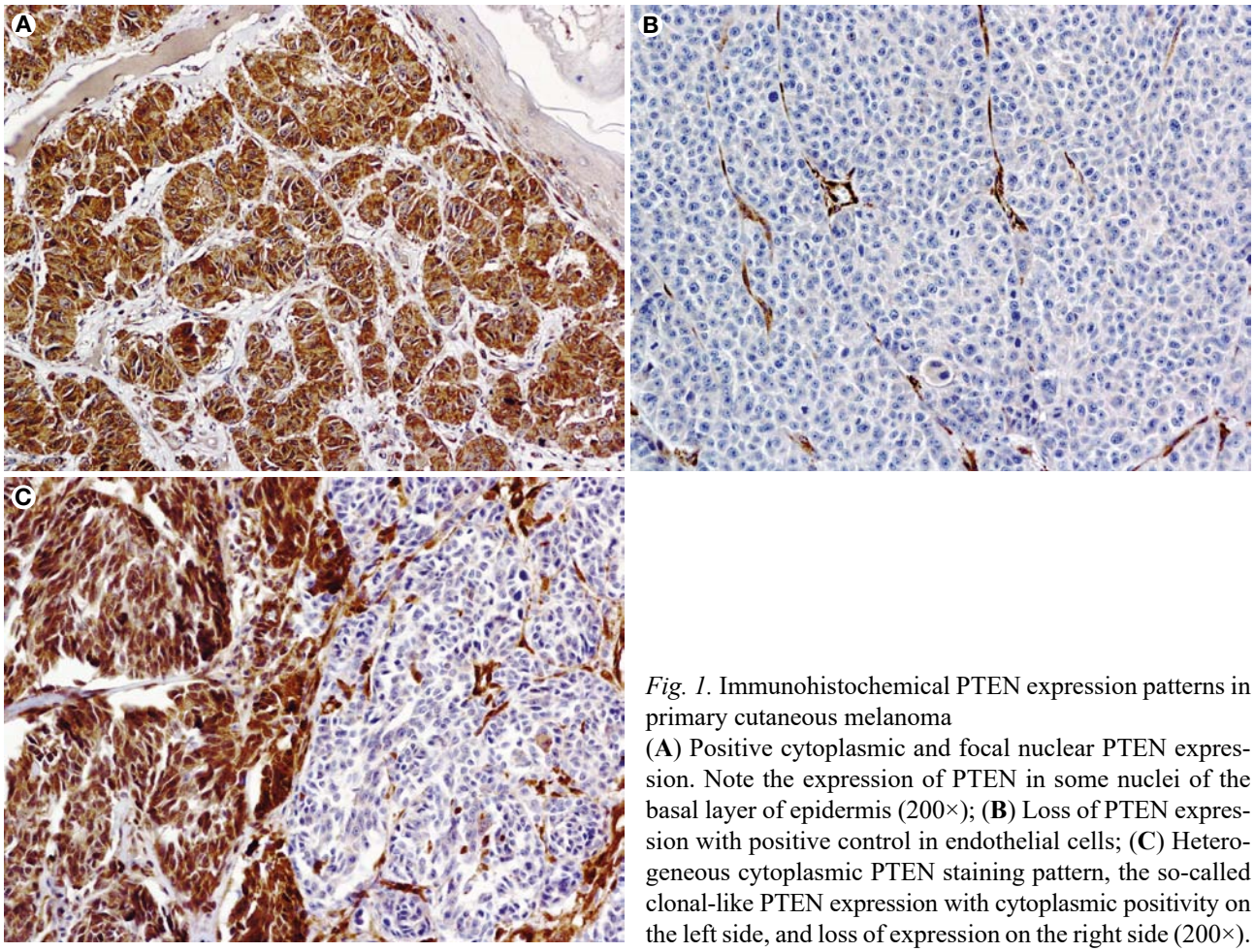

Fig. 1. Immunohistochemical PTEN expression patterns in primary cutaneous melanoma
(**A**) Positive cytoplasmic and focal nuclear PTEN expression. Note the expression of PTEN in some nuclei of the basal layer of epidermis (200×); (**B**) Loss of PTEN expression with positive control in endothelial cells; (**C**) Heterogeneous cytoplasmic PTEN staining pattern, the so-called clonal-like PTEN expression with cytoplasmic positivity on the left side, and loss of expression on the right side (200×).

we were able to evaluate only 19/31 (61 %) cases. Out of the 19 evaluated samples, only five specimens were not older than three years (those were collected between April and August 2017). The reported invalid results were gained from 11 samples in total, all of which were older than six years. In cases with clonal-like PTEN expression, the evaluation was performed in both negative and positive areas of the tumour tissue for comparison (Zhou et al., 2000; Goel et al., 2006; Bucheit et al., 2014; Peng et al., 2016).

The evaluation of the FISH analysis was performed according to different scoring systems used in five different publications (Han et al., 2009; de Campos et al., 2013; Maiques et al., 2014; Picanco-Albuquerque et al., 2016; Lotan et al., 2017), which use different cut-off values (ranging from 24 to 70 %) for evaluation of the normal *PTEN* copy number (characterized by the presence of two CEN10 and two *PTEN* signals), *PTEN* hemizygous detection (LOHe; characterized by the presence of two CEN10 and one *PTEN* signal), *PTEN* homozygous deletion (LOHo; characterized by the presence of two CEN10 and no *PTEN* signal), whole chromosome 10 deletion [del(10); characterized by the presence of one CEN10 and one *PTEN* signal], and polysomy (characterized by the presence of ≥ 3 CEN10 and *PTEN* signals). The cut-off value of ≥ 50 % tumour cells with the respective event was used to calculate the overall score, which was based on the presence of concordant results confirmed by at least three out of five of the aforementioned published criteria. The FISH signals were scored manually (x100 oil immersion) in morphologically intact and non-overlapping nuclei, and a minimum of 50 tumour cells was scored. Signals found in the nuclei of tumour-infiltrating lymphocytes were used as an internal control of the normal *PTEN* copy number.

Isolation of DNA

Sections of formalin-fixed, paraffin-embedded tissue were used for DNA isolation using standard procedures. HE-stained slides were reviewed by a pathologist, and areas for macrodissection, together with an estimation of the tumour cell percentage, were marked. Sections from each sample were deparaffinized in xylene. The DNA was then extracted using the cobas® DNA Sample Preparation kit (Roche, Mannheim, Germany) or automatic isolator MagCore® nucleic Acid Extractor using MagCore Genomic DNA FFPE One-step kit (#MGF-03; RBC Bioscience, New Taipei City, Taiwan).

NGS analysis and biostatistical evaluation

Samples for sequence capture NGS (next-generation sequencing; massive parallel sequencing) were processed using the KAPA HyperPlus kit according to the Seq Cap EZ protocol (NimbleGen, Roche). Target sequences were enriched using a panel of hybridization probes against multiple targets including the whole *PTEN* coding sequence (219 kbp; NimbleGen, Roche). NimbleGen probes were designed using the online software NimbleDesign (parameters for probe stringency were chosen as follows: Probe Selection Database: hg19/GRCh37, Homo sapiens Stringent; Preferred Close Matches: 1; Maximum Close Matches: 5). High stringent probes were designed in order to match only one target to avoid enrichment of homologous sequences. The library was pair-end sequenced by the MiSeq instrument (Illumina, San Diego, CA). NextGENe software (Softgenetics, State College, PA) was used for biostatistical analysis of sequencing data. Nonsynonymous variants in exons and adjacent intronic regions with minimal average coverage 100× and variant allele frequency (VAF) > 10 % were evaluated and manually inspected using an IGV viewer (Broad Institute, UC San Diego, CA) to avoid false-positive variations. The nomenclature follows the rules of the Human Genome Variation Society, and reference sequences used for PTEN were LRG_311 (NM_000314.4, NP_000305.3).

Methylation analysis

Bisulphite conversion of DNA was successfully performed in 71 cases of primary melanoma by using EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. The primers used in the methylation-specific assay were designed according to Garcia et al. (2004); amplicon size for methylated DNA is 71 bp and for unmethylated DNA 78 bp (Gacia et al., 2004). Experiments included non-methylated DNA and universally methylated DNA controls (Human HCT116 DKO Non-Methylated DNA and Human HCT116 DKO Methylated DNA; Zymo Research). PCR was carried out with ZymoTaq™ DNA Polymerase (Zymo Research). The PCR products were separated in 2% agarose gel and the intensities of specific PCR products were evaluated. The methylation pattern of selected PCR products was analysed further by employing a more sensitive detection method using the Fragment Analyzer (AATI, Santa Clara, CA) capillary electrophoresis system and High Sensitivity NGS Fragment Analysis Kit (#DNF-474; AATI).

Statistical analyses

The Statistica software (StatSoft, Tulsa, OK) was used to perform all statistical analyses. Pearson χ^2 test was used to evaluate the association of PTEN IHC groups with clinico-pathological variables. Association of the occurrence of mutations in PTEN (binary response variable: wild-type/mutated) with the clinico-pathological characteristics was also evaluated using Pearson χ^2 test (however, our sample set is limited, as only eight cases carried either pathogenic PTEN mutation detected by NGS or showed loss of heterozygosity (LOH) detected by FISH). One case of histological subtype ALM (PTEN mutated) was excluded from all statistical correlations of the PTEN expression/mutation status with the histological subtype. Time-to-event analysis was performed with three outcomes – disease-free survival (DFS: death from melanoma was considered as a failure), local recurrence-free survival (LFS: the period from primary diagnosis until the first local recurrence), and distant me-

tastasis-free survival (MFS: the period from primary diagnosis until the first distant metastasis). The initial univariate analysis was performed using the log-rank test to determine the differences between IHC groups, and the Kaplan-Meier method was used to compose the survival curves. All performed tests were two-sided and a P-value < 0.05 was considered as significant.

Results

The immunohistochemical evaluation of cytoplasmic staining revealed 20/112 (18 %) cases with loss of PTEN expression and 92/112 (82.1 %) cases with positive PTEN expression, including 11/112 cases (9.8 %) with heterogeneous clonal-like expression. In all but one of the cases with positive PTEN expression, the 2+ intensity staining was dominant (intensity comparable with the intensity of the internal positive control). Surprisingly, one of the 11 cases with clonal-like loss of expression showed positive nuclear PTEN staining within the "clone" population of cells with negative cytoplasmic staining. The evaluation of nuclear staining revealed loss of PTEN expression in 83/112 (74 %) and retained PTEN expression in 29/112 (26 %) cases, including 24/29 (83 %) cases with positivity in 10–49 % of tumour cells and 5/29 (17 %) cases with positivity in ≥ 50 % tumour cells.

The correlations of the cytoplasmic and nuclear PTEN expression with the evaluated clinico-pathological variables are summarized in Table 2. Our data suggest that positive PTEN expression is more commonly found in lower stage melanomas compared to pT3 and pT4 stages, and in those located on the head and extremities compared to those on the trunk.

Survival analyses suggested longer DFS for patients with positive cytoplasmic PTEN expression (group 1+2) in primary melanoma when compared with cases with loss of cytoplasmic PTEN expression (group 0; Fig. 2A). The same trend was observed for nuclear PTEN expression; however, the analysis only showed borderline significance in our data set (Fig. 2B). No significant differences were observed for LFS or MFS (data not shown).

FISH analysis revealed a deletion of *PTEN* in 3/7 (43 %) analysed cases with clonal-like PTEN expression, including two cases with deletion of *PTEN* loci [del(10)] and one case with hemizygous deletion of PTEN (LOHe). Interestingly, the case with LOHe showed immunohistochemical nuclear positivity in the cytoplasmically negative "clonal" focus. In contrast, del(10) was

Table 2. Association between the IHC expression and clinico-pathological variables

IHC	**Cytoplasmic expression group (N)**			**P value**	**Nuclear expression group (N)**		**P value**
Variables	**0** (20)	**1** (81)	**2** (11)		**0** (83)	**1** (29)	
Gender				0.259			0.359
Male	13	49	4		51	15	
Female	7	32	7		32	14	
Age				0.442			0.281
≤ median	8	41	7		39	17	
> median	12	40	4		44	12	
Tumour stage				**0.013**			0.086
pT1 + pT2	0	26	3		18	11	
pT3 + pT4	20	55	8		65	18	
Location				**0.038**			0.141
Trunk	13	29	3		53	14	
Head + Upper + Lower extremities	7	52	8		30	15	
Histological subtype[a]				0.407			0.123
NM	12	47	8		36	8	
SSM	8	34	2		46	21	
Ulceration				0.729			0.287
Yes	8	38	6		41	11	
No	12	43	5		42	18	
Sentinel node positivity[b]				0.750			0.420
Yes	5	11	3		15	4	
No	10	35	7		36	16	

IHC groups for cytoplasmic expression: 0 = negative expression, 1 = positive expression, 2 = positive expression with negative clones (clonal-like PTEN expression). P values are based on Pearson's χ^2 test. [a]One case of ALM was excluded from the statistics. [b]Data not available for all cases.

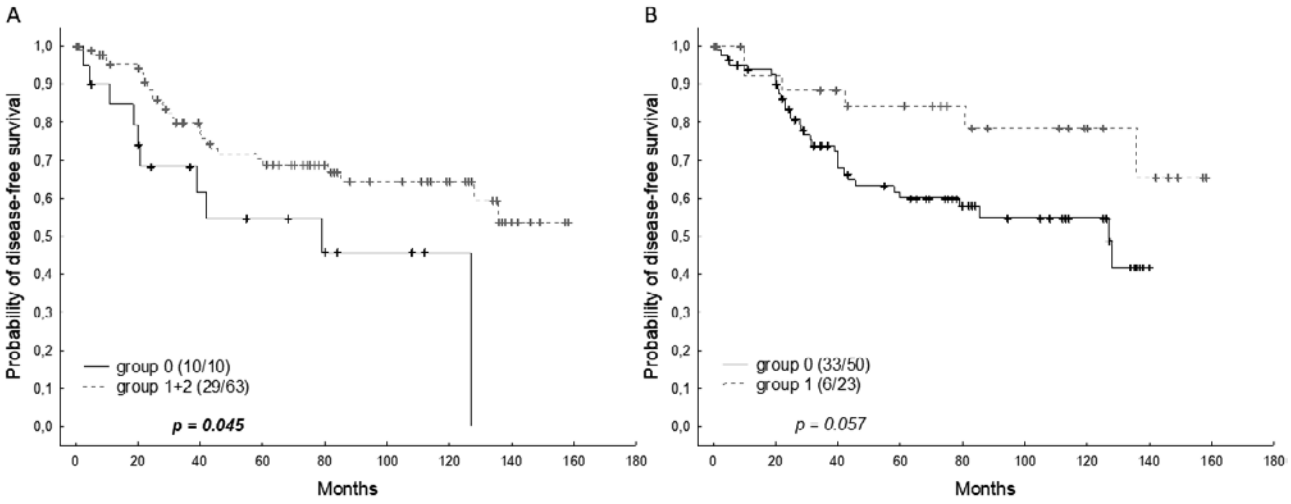

Fig. 2. Correlation of PTEN expression with prognosis
Representative Kaplan-Meier curves for disease specific-free survival (DFS) according to the cytoplasmic (**A**) and nuclear (**B**) expression of PTEN protein evaluated by immunohistochemistry. Group 0 – negative PTEN expression, group 1 – positive PTEN expression, group 2 – clonal-like PTEN expression (only cytoplasmic staining was evaluated). The graphs show marked respective groups with numbers of complete/censored cases in the brackets.

observed in 2/12 (17 %) analysed cases with loss of cytoplasmic and nuclear PTEN expression. We did not observe any homozygous deletion of the *PTEN* loci or polysomy.

The methylation analysis of the PTEN promoter was successfully evaluated in 71 cases. However, no case with promoter methylation was found.

The NGS analysis revealed four *PTEN* variants (classified as pathogenic or splice) in 3/112 (3 %) melanoma cases and one benign variant in one case. However, the frequency of *PTEN* variants could be underestimated. We may have missed several variants due to unequal coverage of *PTEN* in the NGS library. Only 27 % (30/112) of cases had a fully sequenced *PTEN* with sufficient average coverage ≥ 100× in all coding exons. The minimal coverage of several exons, mostly exon 5, was too low (< 100×) to interpret the results, which was caused by advanced DNA fragmentation in a majority of the analysed samples and the high stringency design of probes for the target enrichment during the library preparation. There were somatic pathogenic mutations in exon 6 c.625G>T, p.(Gly209Ter), VAF 14 % (coverage 394×) and in exon 2 c.86_87insAAA, p.(Tyr29delinsTer), VAF 12 % (coverage 216×), detected in one melanoma case (SSM, pT3, located on the trunk, diagnosed in a male patient at the age of 67). We also detected pathogenic *NRAS* mutation c.182A>G, p.(Gln61Arg) in the same patient. No *PTEN* promoter methylation or loss of PTEN expression were found. Another *PTEN* mutation (c.16A>T, p.(Lys6Ter), VAF 43 % (coverage 386×)) was also detected, this one in an NM, pT3, located on the upper extremity, diagnosed in a female at the age of 77. Methylation could not be tested due to the lack of DNA, FISH was not evaluable due to poor tissue quality, and immunohistochemistry showed a loss of PTEN expression. A splice mutation was detected in the consensus splice site in intron 8 c.1026+2T>G, VAF 40 % (coverage 502×), discovered in an NM, pT3, female, diagnosed at 55 years of age. The melanoma showed non-methylated *PTEN* promoter region, positive cytoplasmic expression in 30 % of tumour cells, and a loss of nuclear PTEN expression. FISH was not evaluable. Finally, we also detected a likely benign variant c.883C>G, p.(Leu295Val), VAF 24 % (coverage 309×), and deleted *PTEN* loci, del(10) in one SSM, pT4, trunk, male, diagnosed at 69 years of age. This sample displayed a clonal PTEN expression by immunohistochemistry.

Altogether, seven out of eight cases with some genetic aberrations detected by either NGS and/or FISH also showed aberrant PTEN protein expression (four cases with the loss of PTEN expression and three cases with clonal-like PTEN expression). We did not find any statistically significant correlation between the presence of genetic aberrations and any of the observed clinicopathological variables (data not shown).

Discussion

Since PTEN has distinct functions in the cytoplasm and in the nucleus, we assessed PTEN expression in both of these compartments separately. Cytoplasmic PTEN primarily plays a role in the regulation of PI3K/PIP3 signalling, and influences cancer cell growth, survival, angiogenesis, and metabolism (Song et al., 2012). Nuclear PTEN functions as a tumour suppressor in a PI3K-independent manner through its protein phosphatase activity, causing inhibition of migration or inducing cell cycle arrest (Milella et al., 2015). However, it can also act in a phosphatase-independent way, influ-

encing the regulation of chromosome stability, DNA repair, and apoptosis (Dillon and Miller, 2014; Milella et al., 2015).

We found a loss of cytoplasmic PTEN expression in 20/112 (18 %) cases and clonal-like loss of expression in other 11 (9.8 %) cases, altogether comprising 31 samples (28 %) with aberrant expression. Loss of nuclear expression was found in 83 (74 %) cases (Table 2). Our results are in concordance with most of the previously published studies evaluating primary cutaneous melanomas, which used the same antibody clone (Whiteman et al., 2002; Goel et al., 2006; Bucheit et al., 2014). Different results were found in metastatic melanomas, where about 65 % of cases (Whiteman et al., 2002) showed absent or decreased cytoplasmic expression and almost all samples showed none or decreased nuclear expression (Zhou et al., 2000; Whiteman et al., 2002).

Several studies reported somewhat ambiguous results with a higher proportion of positivity in primary or metastatic melanomas (Deichmann et al., 2002; Mikhail et al., 2005; Giles et al., 2019). The inconsistent results are probably caused by the use of different primary antibodies. Another matter is also the subjectivity of the scoring systems, which are based on a comparison of staining intensity with the adjacent normal cells (vascular endothelium), and PTEN heterogeneity within tumours (Dillon and Miller, 2014). The PTEN antibody (D4.3) used in the study of Giles et al. (2019) showed a higher sensitivity for the detection of PTEN than the 6H2.1 antibody used by us and other aforementioned authors. The discrepant results published in the study by Mikhail et al. (2005) can be explained by the application of a different scoring system and antibody.

Our data showed that the loss of immunohistochemical PTEN expression is more common in higher stages, which is in concordance with others (Goel et al., 2006). Loss of PTEN expression is also more common in melanomas located on the trunk when compared to the head and extremities (Whiteman et al., 2002). The absence of cytoplasmic PTEN expression was also associated with a worse DFS ($P = 0.045$; Fig. 2a). However, the statistical significance of the loss of nuclear staining in our study was only borderline ($P = 0.057$). We have not found any statistically significant outcome regarding LFS and MFS. Other studies analysing the correlation between PTEN expression and disease outcome are inconsistent and sparse. One study suggests that partial PTEN loss is associated with worse overall survival (OS) (Giles et al., 2019). Another study that focused on metastatic melanomas showed that only a complete loss of PTEN expression is associated with shorter OS (Bucheit et al., 2014). Others did not confirm any significant association between the PTEN expression and survival (Mikhail et al., 2005). One explanation of this discordance may lie in the divergence between the analysed sample sets and the different used antibodies.

Only a few studies performed a cytogenetic analysis in melanomas to date, and those used centromeric probes for the chromosomes (1, 4, 6-7, 9-12, 15, 17-18, X, and Y) and a midisatellite probe localized in 1p36, but no study used the *PTEN* locus-specific FISH probe in cutaneous melanomas (Poetsch et al., 1998a, b). One study of nine cases of uveal melanoma cell lines did not detect any *PTEN* mutations or cytogenetic abnormalities involving chromosome 10q23, and the authors suggest that *PTEN* alterations probably do not play a role in the pathogenesis of uveal melanoma when compared to cutaneous melanoma (Naus et al., 2000).

The loss of PTEN *loci* was detected in 2 of 12 (17 %) analysed cases with a loss of PTEN expression (cytoplasmic and nuclear) and in 3/7 (43 %) cases with clonal-like PTEN expression. Interestingly, one case with a heterozygous PTEN deletion (LOHe) showed immunohistochemical nuclear positivity in the cytoplasmically negative "clonal" focus. Since this is only an isolated case, it is impossible to find a relevant explanation for this observation. Altogether, the FISH analysis revealed a deletion of *PTEN* in 5 out of 19 (26 %) analysed cases. Additionally, four pathogenic, likely pathogenic, or splice *PTEN* variants were detected by NGS in three different samples out of the 112 analysed cases. All the cases were associated with a loss of nuclear PTEN expression, and in two cases there was also a loss of cytoplasmic expression. One case with a retained PTEN cytoplasmic expression showed staining in 30 % of tumour cells. Altogether, there were eight cases with *PTEN* genetic aberration detected by either FISH or NGS, which included one case with positive expression, four cases with a loss of expression, and three cases with clonal-like *PTEN* expression. We did not find any statistically significant association between the detected genetic aberrations (FISH, NGS) and the clinico-pathological variables (data not shown). The agreement between the observed aberrant PTEN expression and detected genetic aberrations by FISH was described by others in prostatic or endometrial cancer (Han et al., 2009; Maiques et al., 2014; Picanco-Albuquerque et al., 2016; Lotan et al., 2017). Another study described hemizygous or homozygous *PTEN* deletion in 40 % and 6 % renal cell carcinomas, respectively. Nevertheless, no correlations with immunohistochemical PTEN expression were examined in this study (de Campos et al., 2013). Homozygous deletion of *PTEN* loci or polysomy were also observed in prostatic cancer cases (Han et al., 2009; Picanco-Albuquerque et al., 2016; Lotan et al., 2017). In contrast, no homozygous alterations were detected in our study.

Epigenetic changes can explain loss of the PTEN function; however, the precise role of DNA methylation in melanoma is still unclear (Dillon and Miller, 2014). We did not observe any *PTEN* promoter methylation in our sample set. Data regarding *PTEN* promoter methylation in melanoma is quite inconsistent in the literature. Our results are in agreement with the data derived from The Cancer Genome Atlas Network (TGCA, http://www.cbioportal.org/), which included a cohort of skin cutaneous melanomas (Cancer Genome Atlas, 2015). The TCGA melanoma DNA methylation data included

473 melanomas. Methylation of the *PTEN* promoter was found in only 0.6 % of melanomas (3/473). Our results together with the methylation analysis data derived from TCGA suggest that *PTEN* promoter methylation is not common in primary cutaneous melanomas. However, the results of some other studies are rather different. Some studies reported varying rates of methylation in melanomas or melanoma cell lines (23–62 %) (Mirmohammadsadegh et al., 2006; Lahtz et al., 2010; de Unamuno Bustos et al., 2018). Mirmohammadsadegh et al. (2006) have found *PTEN* promoter methylation in 26 % or 62 % of circulating DNA isolated from the sera of patients with primary or metastatic melanoma, respectively. Others found methylation in 24 % of primary cutaneous melanomas (Mirmohammadsadegh et al., 2006; de Unamuno Bustos et al., 2018). The association of PTEN methylation with higher age, Breslow thickness, advanced stage, and acral localization has also been shown (de Unamuno Bustos et al., 2018). The PTEN promoter methylation has been found to be an independent prognostic marker in cutaneous melanoma associated with poor survival (Roh et al., 2016; de Unamuno Bustos et al., 2018). Micevic et al. (2017) confirmed the correlation between methylation and protein expression and found an association with increased risk of death by Cox regression analysis. In contrast, Giles et al. (2019) did not find any relationship between these variables.

Concurrent loss of *PTEN* with activating *BRAF* mutation is responsible for the acquired resistance of melanoma to BRAF inhibitors (Shi et al., 2014; Leonardi et al., 2018). However, the loss of PTEN function by itself does not preclude an antitumor response to BRAF inhibitors, since that is only one of the mechanisms responsible for the resistance, and it is therefore likely that other co-occurring events cooperate in the development of resistance. Brown et al. (2017) proposed that the effects of β-catenin signalling in melanoma cells differ depending on PTEN expression. The loss or absence of PTEN expression corresponds with increased invasion, and retained PTEN staining is associated with reduced invasion in response to β-catenin signalling (Brown et al., 2017). The authors suggest that the pro-invasive effects of WNT/β-catenin signalling are potentially independent of metabolic reprogramming. Their results suggest that the state of PTEN expression could be a potential biomarker for the implementation of WNT inhibitors in melanoma. Abbots et al. (2014) provided the evidence that PTEN deficiency is not only a promising biomarker in melanoma, but can also be targeted by a blockage of DNA base excision repair (by APE1 inhibition) and as such could represent a potential target for targeted therapy of PTEN-deficient melanomas. Some recent studies demonstrated that the PTEN expression status also plays an important role in determining the response to MEK inhibition in melanoma, and a loss of PTEN hinders the anti-tumour activity of MEK inhibitors in preclinical models (Ciuffreda et al., 2012; Milella et al., 2015).

In conclusion, the results of our study showed that the loss of cytoplasmic PTEN expression was significantly associated with higher stage, location of melanoma on the trunk, and worse DFS. This is in concordance with some of the previously published studies. Concerning molecular findings, we identified eight cases with aberrations detected by FISH or NGS, but no case with methylation of the *PTEN* promoter. These findings are in agreement with the results of TCGA study, but not with several other studies, and further research into this topic is needed. Despite the limitation in the number of fully analysed cases, our data showed a poor concordance between the aberrant IHC pattern and the presence of *PTEN* mutation or aberration in the *PTEN* loci, which suggests that IHC is an inappropriate screening method for the detection of genetic aberrations. Nevertheless, the loss of PTEN expression as detected by immunohistochemistry can be clinically significant. However, studies are needed to assess the significance of PTEN analysis, either as a potential biomarker of response to targeted therapy (BRAF inhibitors, MEK inhibitors, WNT inhibitors, blockage of DNA base excision repair, and others), or directly in targeted therapy using PTEN modulators in the future.

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