Original Article

Comparison of Subtypes of Hepatocellular Adenoma to Hepatocellular Carcinoma and Non-Neoplastic Liver Tissue in Terms of PTEN Expression

(PTEN / hepatocellular adenoma / hepatocellular carcinoma / carcinogenesis / hepatocyte)

G. SZPARECKI, T. ILCZUK, N. GABZDYL, B. GÓRNICKA

Department of Pathology, Medical University of Warsaw, Warsaw, Poland

Abstract. *PTEN* is a tumour suppressor gene whose loss of function has been found to be present in a variety of neoplasms, both benign and malignant. In hepatocellular carcinoma (HCC), loss of PTEN is associated with poorly differentiated cancer, advanced clinical stage and tendency to recur. The extent and meaning of PTEN loss in hepatocellular adenoma (HA), one of the precursor lesions for HCC, has not yet been analysed. The aim of the present study was to evaluate the possible loss of PTEN expression in HA in the wider context of hepatocarcinogenesis. Immunohistochemical analysis of PTEN expression was performed in non-neoplastic liver tissue, HAs and HCCs. It has been found that the loss of PTEN was markedly present in poorly differentiated HCC, whereas well to moderately differentiated HCC showed similar levels of PTEN expression to non-neoplastic liver. HAs presented as a heterogeneous group, with loss of PTEN observed in the inflammatory and *HNF1A*-mutated subtype and relatively intact PTEN expression in HA with nuclear β-catenin overexpression. This suggests that the loss of PTEN might occur both in HA and HCC, constituting different outcomes of the same molecular lesion in the various contexts of malignant or benign neoplasms.

Introduction

Phosphatase and tensin homologue (PTEN), encoded by the *PTEN* gene, is one of the most important tumour suppressor proteins. In non-neoplastic cells the protein regulates cell cycle, preventing too rapid divisions of the cell (Chu et al., 2004). Inactivation of PI-3-kinase-dependent signalling is the mechanism through which PTEN inhibits cell proliferation, growth and survival (Leslie et al., 2004).

The loss of function of PTEN has been found to be present in a variety of neoplasms, both benign and malignant. It has long been known that mutations in the *PTEN* gene cause a clinical syndrome known under its eponymous name, Cowden syndrome (Lloyd et al., 1963). As revealed by wide-range sequencing (Lynch et al., 1997), inactivation of the PTEN protein in patients with Cowden syndrome was caused by point mutations altering a single amino acid in the protein chain or (more often) yielding a stop-codon and causing premature translation termination and production of a truncated protein.

As far as PTEN dysfunction is concerned, it has been found that both the spectrum of clinically observed neoplasms and the underlying molecular mechanisms leading to disruption of the PTEN activity evince far more variety in sporadically occurring tumours than in the Cowden syndrome. Not only point mutations, but also hypermethylation of the promotor region (Siddiqui et al., 2016) and silencing miRNA activity (Meng et al., 2007) may lead to the loss of PTEN function.

As has been already noted, the variety of tumours with proved functional loss of PTEN far exceeds those commonly identified as constituents of Cowden syndrome. Loss of PTEN has been reported in osteosarcoma (Xi et al., 2017), squamous cell cancer (Lu et al., 2017) and gallbladder cancer (Tekcham et al., 2017) to name only a few. Apart from the self-explanatory role of PTEN in cell cycle regulation and resulting unhemmed proliferation in case of its loss, the lack of PTEN function might influence cancer cells in a plethora of other ways. Down-regulation of PTEN expression promotes epithelial-mesenchymal transformation in keratinocytes (Yan et al., 2017) and induces angiogenesis, when found in the epithelium (Unseld et al., 2015). Similar results were obtained in the malignant neoplastic setting (Ye et al., 2015; Zhang et al., 2016).

Hepatocellular carcinoma (HCC) is one of the tumours in which loss of PTEN has been documented. In about 40 % of HCC cases, PTEN expression was lacking or diminished, and this event is considered to be a poor prognostic factor, associated with poorly differentiated cancer, advanced clinical stage and tendency to recur (Hu et al., 2003). The role of PTEN in hepatocellular carcinoma drew enough attention to validate its considerations as a potential target for novel therapeutic approaches (Augello et al., 2016).

Despite its well-documented role in the development of HCC and non-neoplastic liver diseases, especially in poorly differentiated tumours, there were no attempts as yet to assess its expression in pre-cancerous lesions with risk of malignant transformation into HCC, such as hepatocellular adenoma (HA). It has been estimated that about 5–15 % of HAs may undergo this process, which might be one of the main pathogenic explanations for HCC occurrence in non-cirrhotic liver (Liu et al., 2014). The risk for malignant transformation depends on the molecular subtype of HA, with β-catenin-activated subtype being associated with the highest risk (Agrawal et al., 2015). HAs have not be extensively researched as far as their molecular provenance is concerned. Apart from identifying three distinct molecular subtypes, little is known about the possible molecular changes causing normal hepatocytes to proliferate and create a distinct benign neoplastic entity. The molecular changes mentioned as a basis for distinguishing subtypes are: *IL6ST* mutation (characteristic of inflammatory subtype), *HNF1A* mutation and Wnt/β-catenin pathway disruptions (Calderaro et al., 2016). This simple division has been recently refined by noting the overlapping subtype of β-IHCA (hepatocellular adenoma sharing features of inflammatory type with β-catenin pathway disruptions) and unclassified HA, lacking features of any of the previous subtypes (Roncalli et al., 2016).

In contrast, adenomas in other locations that are known for their propensity to malignant transformation, such as colon adenomas, have been researched in the light of PTEN expression. Colorectal adenomas, in general, showed unchanged PTEN expression compared to non-neoplastic tissue – only 8 % of these lesions showed loss of PTEN (Bendib et al., 2015). Nonetheless, the PTEN protein is believed to suppress adenoma development even in the presence of *APC* mutation (Marsh et al., 2008). On the other hand, serrated polyps in animal models showed decreased PTEN expression (Davies et al., 2014). It is therefore postulated that at the early stages of carcinogenesis, the PTEN function is preserved (in adenomas), whereas its loss in serrated polyps (Pap et al., 2015) and carcinomas (Molinari and Frattini, 2013) portends dysregulation and worsening of molecular condition of the tissue. Therefore, loss of PTEN might be placed in the sequence of genetic and epigenetic alterations during progression from colorectal adenoma to carcinoma somewhere in the middle of the process, just before final mutations occur, which are a hallmark of malignant progression itself.

The aim of the present study was to study the possible loss of PTEN expression in hepatocellular adenoma. The wider context of non-neoplastic hepatocytes of the control group and malignant tumours of hepatocyte origin was taken into account. On the other hand, the molecular diversity of HAs was analysed, with selection of benign tumours with β-catenin activation and comparison of PTEN expression in this subtype with other HAs taken as a whole.

This analysis could fill the gap that exists in our knowledge about carcinogenesis in hepatocytes. Although a plethora of molecular changes have been identified as extant in HCC, their relative importance and sequence of events (similar to that of the colon adenoma-adenocarcinoma sequence) have not been unequivocally established. Analysis of the pattern of molecular disruptions in hepatocellular adenoma could reveal which changes are most likely to occur early in carcinogenesis, and which are only a late event characteristic of high-grade HCC.

Material and Methods

Ethics statement

The present study was approved by the Bioethics Committee of Medical University of Warsaw (AKBE/ 61/14).

Tissue specimens

Tissue samples were obtained from 59 patients, who were divided into three groups on the basis of histopathological diagnosis: 20 patients (4 males and 16 females, average age 43 years, range 17–69; average tumour diameter 6.15 cm, range 2–16 cm) diagnosed with hepatocellular adenoma, 19 patients (14 males and 5 females, average age 52 years, range 28–70; average tumour size 9.95 cm, range 4–18 cm) diagnosed with hepatocellular carcinoma G3, 9 patients (6 males and 3 females, average age 62 years, range 48–73, average tumour size 6.52 cm, range 3.5–18 cm) diagnosed with hepatocellular carcinoma G1 or G2, and 11 patients (4 males and 7 females, average age 49 years, range 20–63) without any significant pathology of the liver. The exclusion criteria for the last group were: histopathologic diagnosis of a liver neoplasm, liver cirrhosis or fibrosis, massive steatosis (involving over 20 % of hepatocytes) or inflammation, HBV or HCV infection.

The diagnosis of HA (as contrasted with HCC G1) was made based on the histological criteria both in standard haematoxylin-eosin (H&E) staining and using additional immunohistochemical and histochemical stains. In H&E staining, criteria for HA diagnosis were lack of cytologic atypia and absence of architectural abnormalities (Shafizadeh and Kakar, 2011). All of our cases diagnosed as HA were negative for glypican-3 (Choi et al., 2017) and evinced normal reticulin meshwork as revealed by reticulin-Gomorri stain (Dominguez-Malagon and Gaytan-Graham, 2001).

The grading of HCC cases, i.e., their subdivision into G1, G2, or G3 histological grade was performed as part of a routine diagnostic process according to the criteria presented in Table 1.

Immunohistochemistry

Four μm-thick formalin-fixed paraffin-embedded sections were deparaffinised in xylene and alcohols. All immunohistochemistry reactions were performed in a Leica BOND-III automated stainer (Leica Biosystems, Nussloch, Germany). Heat-induced epitome retrieval was performed at pH = 8.0 for 40 min (for reaction with anti-PTEN antibody) or at pH = 9.0 for 20 min (for anti-β-catenin antibody). Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide for 30 min. After this step, sections were placed in 5% normal horse serum (NHS, Jackson Immunoresearch, West Grove, PSA) in order to reduce unspecific binding of the antibody. The anti-human PTEN (monoclonal mouse, cat. No. M3627, Dako, Glostrup, Denmark) antibody was applied in 1 : 100 dilution in 5% NHS, or anti-β-catenin antibody (monoclonal mouse, cat. No. M3539, Dako) was applied undiluted kept at room temperature for 45 or 30 min, respectively. In order to detect primary antibodies, secondary antibodies were used, directly conjugated with peroxidase particles (cat. No. DS9800, Leica Biosystems) according to manufacturer's instructions. The reaction was presented using 3,3'-diaminobenzidine as chromogen. Cell nuclei were exposed in contrast staining with haematoxylin.

The slides stained with anti-PTEN antibody were scanned in their entirety using NanoZoomer-XR Digital slide scanner C12000 (Hamamatsu, Naka-ku, Japan) at 40x magnification in a single layer. The slides stained with anti-β-catenin antibody were analysed under light microscope Ci Eclipse (Nikon, Tokyo, Japan) to determine the presence of nuclear reaction in order to classify each adenoma case as Wnt/β-catenin dysfunction-associated type (nuclear expression of β-catenin present) or tumour without Wnt/β-catenin pathway dysfunction (no nuclear expression of β-catenin, see Fig. 1A,B).

Statistical analysis

The PTEN immunoreactivity was analysed in the full-slide high-quality images obtained from a Hamamatsu scanner. One hundred cells from each tumour were counted in every virtual slide. The presence and the intensity of staining were both included into the analysis. A three-point scale was applied to diversify the intensity of immunoreaction, from a high (3 points), through me-

dium (2 points) to weak (1 point) value. The number of cells evincing high or medium intensity of immunoreaction was calculated as a percentage of all cells and analysed further. Since the data obtained did not follow normal distribution (as assessed by Shapiro-Wilk test), we used Kruskal-Wallis ANOVA and U Mann-Whitney test to analyse the differences between groups in terms of the PTEN expression score. P levels under 0.05 were deemed statistically significant.

Results

PTEN expression in hepatocellular adenoma depending on its molecular subtype

Our study revealed that in our group of 20 hepatocellular adenomas, 10 could be classified as Wnt/β-catenin pathway dysfunction based on their nuclear β-catenin overexpression (see Fig. 1A,B).

Hepatocellular adenomas with concurrent overexpression of β-catenin in the nucleus, and therefore presumably belonging to the Wnt/β-catenin pathway dysfunction subtype, showed significantly higher percentage of cells with strongly and moderately positive PTEN staining (median 11 %, range 0–64 %) than adenomas belonging to the remaining two subtypes (inflammatory and *HNF1A*-mutated; median 0 %, range 0–1 %). The result was statistically significant at P = 0.009 (Z = –2.82, see Fig. 1D, Fig. 2). The effect could be described as a strong one, as described by the *r* coefficient, which equalled 0.64.

PTEN expression in malignant and benign liver neoplasms as compared to non-neoplastic liver tissue

The percentage of cells with strongly and moderately positive PTEN staining was highest in non-neoplastic liver tissue (median 1 %, range 0–70 %) and well to moderately differentiated HCC (median 8 %, range 0–62 %). Poorly differentiated HCC showed a median percentage of 0 % (range 0–9 %), whereas hepatocellular adenomas as a whole showed median of 0 % and range between 0 and 64 %. The results were statistically significant at P = 0.01; $H(3)$ = 11.25 (see Fig. 1E,F, Fig. 3). Post-hoc analysis using Bonferroni correction for multiple comparisons showed that poorly differentiated HCC evinced significantly (P = 0.04) lower levels of PTEN expression than well-differentiated HCC. On the other hand, the PTEN expression level in HA taken as a

Table 1. Criteria for histological grading of HCC used in the study (modified from Schlageter et al., 2014)

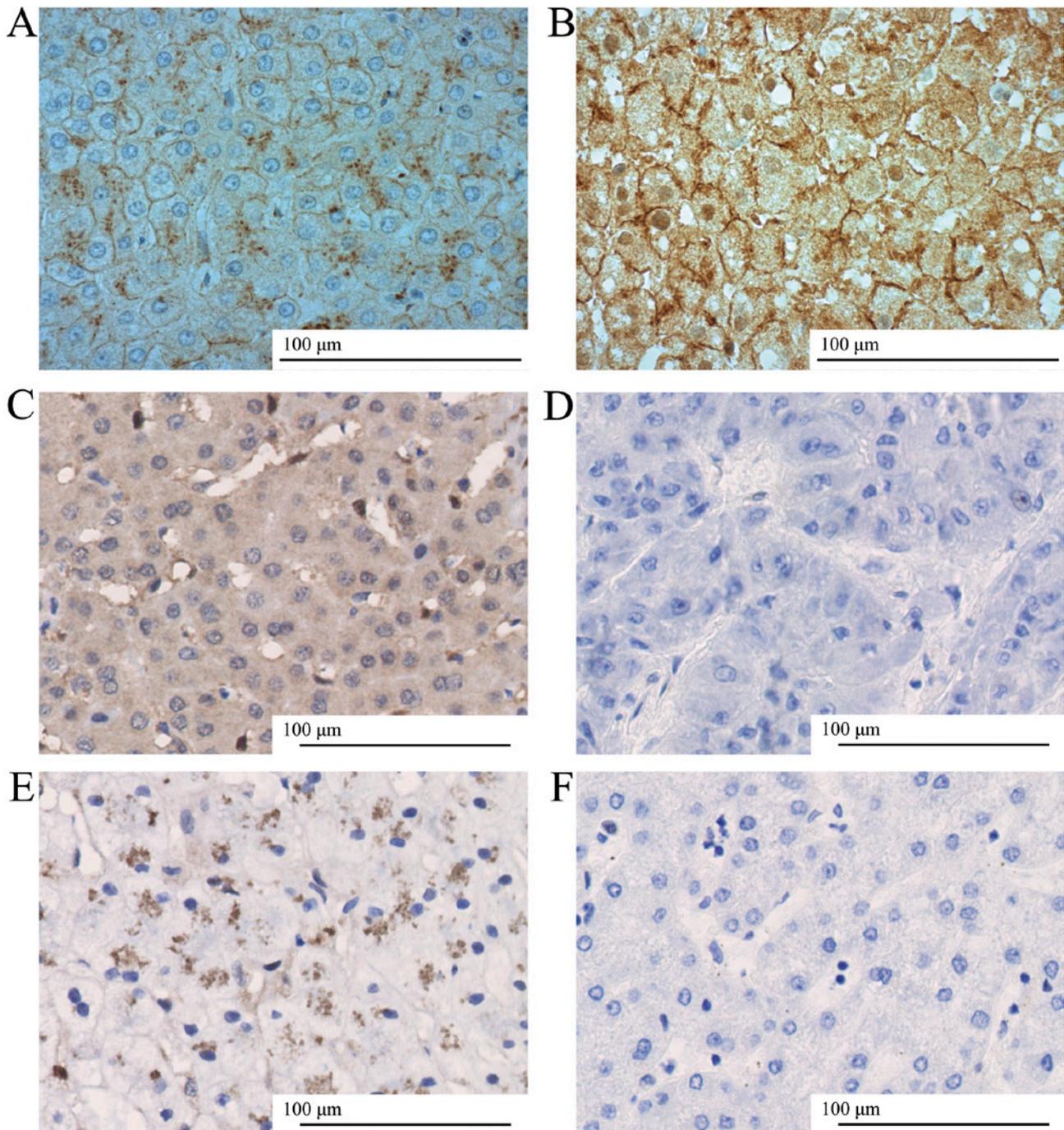| Grade	Microscopic features
G1	Small tumour cells, resembling normal liver in their shape and trabecular arrangement
G2	Small degree of nuclear irregularity, hyperchromatism
G3	Prominent nuclear pleomorphism, marked hyperchromatism, anaplastic cells

Fig. 1. Representative slides demonstrating immunohistochemical reactions (40× magnification). Hepatocellular adenoma without (**A**) and with nuclear expression of β-catenin (**B**). Immunohistochemical staining with anti-PTEN antibody (**C-F**): well-differentiated hepatocellular carcinoma (**C**), poorly differentiated hepatocellular carcinoma (**D**), hepatocellular adenoma with (**E**) and without (**F**) nuclear expression of β-catenin.

whole and in non-neoplastic liver tissue was heterogeneous and spanned an overlapping range covering the range encountered in HCC. The effect could be described as a moderate one, based on $\varepsilon^2 = 0.20$.

Discussion

In our study we analysed expression of the PTEN protein in the studied tissues in terms of the percentage of moderately and strongly positively staining cells. We believe that this population represents the cells retaining PTEN expression beyond any possibility of deletion or missense mutations. Taking into account weakly positive cells could pose the danger of erroneous consideration of unspecific staining. It is possible that cells (especially those in normal liver not exposed to detrimental factors) without any alterations of the *PTEN* gene could express the protein only to a limited degree (correspond-
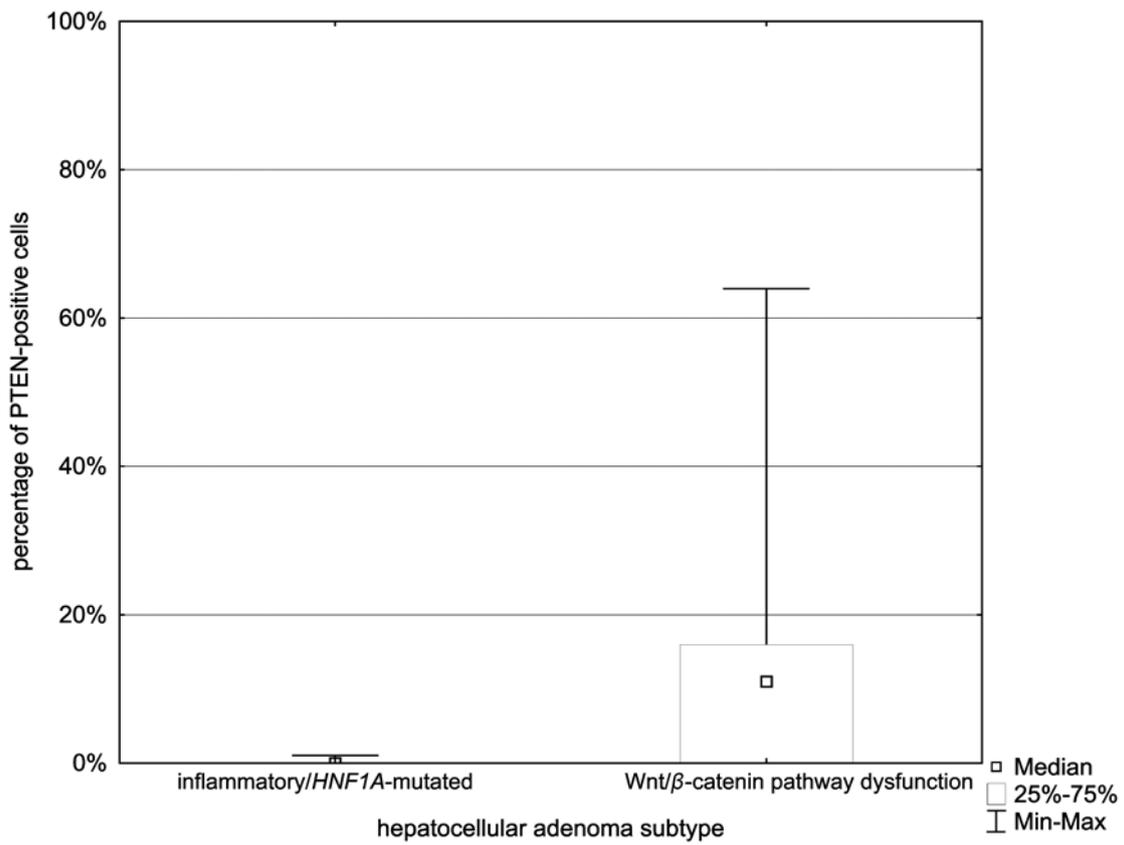
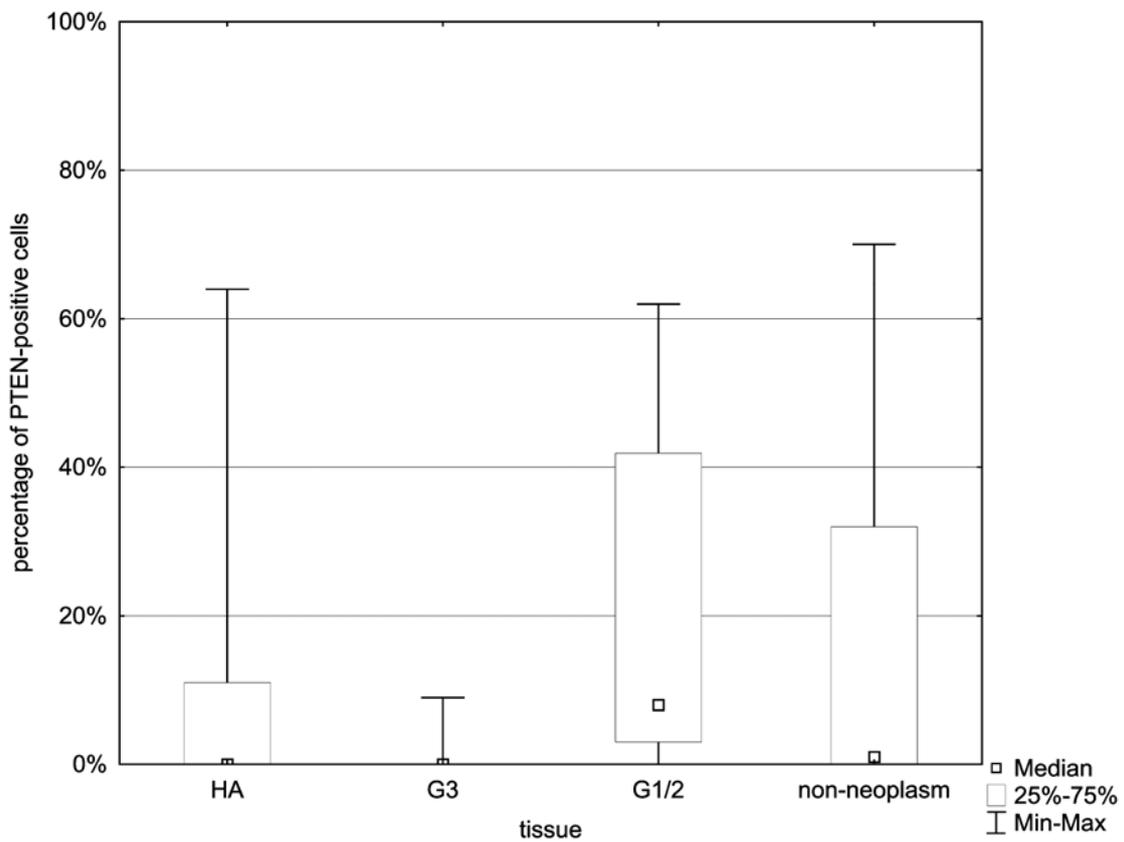*Fig. 2.* Comparison of PTEN expression in hepatocellular adenoma subtypes

Fig. 3. Comparison of PTEN expression in non-neoplastic and neoplastic liver tissues

ing to weak immunohistochemical reaction). This could lead to underestimation of the percentage of cells with extant PTEN function; however, we believe that erring on the side of a conservative and cautious approach to the IHC reaction yield is better than omitting the possible loss of PTEN. This approach should not bias the whole study, since all groups have been treated uniformly. Our main aim was to analyse hepatocellular adenomas in the context of other neoplastic lesions and as far as their molecular subtype is concerned; therefore, we needed higher sensitivity in the detection of PTEN deletion. This should not have any significant influence on the PTEN status in neoplastic tissues, since loss of PTEN should be unequivocal, even at the cost of underestimating PTEN expression in non-neoplastic liver.

In our study, the PTEN expression was lowest in poorly differentiated HCC. This finding is in line with previously published results associating high histological grade of tumours with the loss of PTEN. The said loss may occur in moderately and well-differentiated tumours as well, however to a lesser extent, which was also the case in our study. The most interesting aspect in our study was the position of HAs on the background of other liver tumours and non-neoplastic conditions. As far as this comparison is concerned, PTEN expression levels in benign hepatocyte tumours spans a range overlaying both these encountered in non-neoplastic liver tissue and HCC; however, the median percentage of cells strongly positive for PTEN tends to be more similar to non-neoplastic and well-differentiated HCC. This could lead to the conclusion that loss of PTEN is not an early event in hepatocarcinogenesis and occurs only late during the process, when the malignancy is already established and further dedifferentiation is on its way.

However, the picture is more complicated when we take a look at the subtypes of HA, especially when we take into consideration dysfunction of the Wnt/β-catenin pathway. This distinction yields two disjoined groups of HAs, which, as has been stated in the Introduction, differ in terms of their molecular background and malignant transformation risk. In our study we found that they differ in the extent to which PTEN expression is lost as well.

Counter-intuitively, HAs without nuclear β-catenin overexpression shared loss of PTEN with poorly differentiated HCCs, whereas the subtype with marked dysfunction of the Wnt/β-catenin pathway resembled more well to moderately differentiated malignant tumours. The reasons for this seemingly less probable result could not be stated with any amount of certainty, since abundant interplays between various molecular pathways, their constituent proteins and genetic underpinnings have been elucidated neither in HCC nor in HA. Nonetheless, we postulate that the PTEN loss in HAs is a distinct molecular event from that in advanced and dedifferentiated liver carcinoma.

In conclusion, our results showed that loss of PTEN is rather a late event in hepatocarcinogenesis, markedly present in poorly differentiated HCCs, however observed only to a limited degree in well and moderately differentiated HCCs and HAs. It should be noted that HAs are heterogeneous as far as their molecular background is concerned, and this variety could be observed in terms of PTEN expression as well. Loss of PTEN, similarly to that observed in poorly differentiated HCCs, could be noted in HAs without nuclear β-catenin overexpression, whereas the Wnt/β-catenin dysfunction subtype showed no significant loss of PTEN compared with non-neoplastic liver tissue and well to moderately differentiated HCC. We therefore suppose that the effects of loss of PTEN could vary, and this variety in the outcome could be traced back to the different molecular settings of malignant or benign tumours. We propose that the loss of PTEN might occur both in HA (encompassing inflammatory and *HNF1A*-mutated subtypes) and HCC (in poorly differentiated tumours). However, this should not be taken as a proof for the possible chronological link and background for the HA-HCC sequence. It should rather be supposed that this finding reflects the heterogeneous effects of PTEN dysfunction, depending on the molecular context of microenvironment in the cell, especially other mutations and epigenetic changes differentiating between benign and malignant neoplasia.

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