Original Article

Expression of c-MET Protein in Various Subtypes of Hepatocellular Adenoma Compared to Hepatocellular Carcinoma and Non-Neoplastic Liver in Human Tissue

(c-MET / hepatocellular adenoma / hepatocellular carcinoma / β-catenin)

G. SZPARECKI, T. ILCZUK, N. GABZDYL, E. STOCKA-ŁABNO, B. GÓRNICKA

Department of Pathology, Medical University of Warsaw, Poland

Abstract. Hepatocellular adenoma (HA) is a benign neoplasm of the liver, whose aetiopathogenesis is little known. Newest research allowed dividing all cases into three types based on molecular characteristics: inflammatory HA, HA with *HNF1A* mutation, β-catenin-mutated HA. The clinical significance of HA is chiefly due to the possibility of malignant transformation into hepatocellular carcinoma (HCC). The aim of the present study was to immunohistochemically assess the expression pattern and level of c-MET protein in hepatocellular adenoma (taking into account its status of Wnt/β-catenin pathway functioning) and intertwining the results into a wider pattern of expression in non-neoplastic liver and hepatocellular carcinoma of various histological grades. It was found that expression of c-MET in poorly-differentiated HCC was significantly higher than in non-neoplastic liver and well- to moderately-differentiated HCC. The expression in HA was variable and differed between molecular subtypes of this neoplasm: inflammatory and *HNF1A* mutation-associated type are characterized by overexpression of c-MET to an extent comparable with poorly-differentiated HCC, whereas Wnt/β-catenin dysfunction-associated type lacks overexpression, and the amount of c-MET protein accumulated in its cells is similar to the levels in non-neoplastic tissue and well- to moderately-differentiated HCC. These findings suggest that c-MET overexpression in HA is not an early event in hepatocarcinogenesis, but constitutes a divergent molecular pathway leading to neoplastic change compared to overexpression observed in the late stages of tumour progression.

Introduction

Hepatocellular adenoma (HA) is a benign neoplasm of the liver of hepatocyte origin. In contrast to most benign tumours, it is not encapsulated. It is characterized by venous and arterial vascularization and size spanning from 0.5 to 15 cm at the diagnosis. Its cells resemble normal hepatocytes with rare mitotic figures and possible lipid or glycogen accumulation (Bioulac-Sage et al., 2007). Earlier it was believed to occur exclusively in women after oral contraceptive exposure (Edmondson et al., 1976); however, nowadays the histological and molecular variety of these tumours is emphasized. The most up-to-date classification encompasses four groups (Margolskee et al., 2016):

1. The inflammatory type (50–60 % of cases) is characterized by marked inflammation in the tumour. It could be caused by *IL6ST* mutation (encoding gp130), which results in constant dimerization of the gp130 protein activating signal transduction through the JAK-STAT3 pathway (Rebouissou et al., 2009). gp130 overexpression is also possible (Spannbauer and Trautwein, 2009).

2. *HNF1A* mutation-associated type (30–35 % of cases). This mutation might occur sporadically (90 %) or have a germinal origin. Oral contraceptives might contribute to its occurrence in adult age (Cavalieri et al., 2000), whereas germ-line mutation is associated with MODY diabetes and familial liver adenomatosis (Reznik et al., 2004). Regardless of the cause, inactivating mutations of the *HNF1A* gene lead to expression of dysfunctional HNF-1α protein, which causes increased lipogenesis and hepatocyte proliferation. Furthermore, dysfunction of the HNF-1α protein silences expression of fatty acids binding protein (L-FABP) and increases lipid accumulation in hepatocytes (Rebouissou et al., 2007).

3. Wnt/β-catenin dysfunction-associated type (10–15 % of cases). Adenomas of this type develop on the basis of *CTNNB1* gene mutations, faulty Wnt protein activation or *APC* mutations. All these mechanisms result in constitutive Wnt/β-catenin pathway activation. This pathway plays a role in differentiation, prolifera-

tion, adhesion and regeneration of the cells (Monga et al., 2003).

4. Unclassified type – adenomas included in this group cannot be classified into any of the preceding types due to the lack of expression of any characteristic markers mentioned above.

The clinical significance of hepatocellular adenomas consists in causing tumour mass symptoms and the possibility of complications, such as rupture with following internal haemorrhage and progression to hepatocellular carcinoma (HCC). The risk of such consequences occurring depends on the type of the tumour – bleeding is associated with inflammation, whereas the probability of malignant transformation is highest in the Wnt/β-catenin dysfunction-associated type (Van der Borght et al., 2007). Nonetheless, it should be stressed that not only this type of HA might transform into HCC (Zucman-Rossi et al., 2006), regardless of other risk factors, such as infection by hepatotropic virus or liver cirrhosis (Kim et al., 2009). Malignant transformation occurs more often in men than in women (Farges et al., 2011) and is associated with tumour diameter of over 4 centimetres (Bioulac-Sage et al., 2007). HCC foci present either as a macroscopic nodule of over 1 centimetre or multiple microscopic nests (Katabathina et al., 2011). Further diagnostic significance of HA stems from its close histological resemblance to well-differentiated HCC (of G1 grade), which might make differential diagnosis problematic.

Hepatocellular carcinoma is one of the most common malignant neoplasms (Llovet et al., 2003). The morbidity factor is constantly increasing due to the prevalence of HBV and HCV. Despite numerous efforts to increase the 5-year survival rate, the prognosis is still bad to dismal. Molecular biology played a key role in elucidating the basis of therapeutic difficulties confronting the clinicians. The reason thereof is a great variety of molecular pathways accompanying HCC development, which on the one hand make creating therapy guidelines difficult, on the other, however, give hope for molecular targeted therapy (Tanaka and Arii, 2012) and laboratory tests to detect early stages of cancer progression (Bertino et al., 2012).

One of the various molecular pathways well characterized in HCC transformation and progression is c-MET hyperactivation. This protein encodes a receptor for hepatocyte growth factor (HGF). Its activation stimulates cells to break inter-cellular bonds and migrate. The disruption of signal transduction and constant activation of the receptor is an important event taking place in a variety of neoplasms, including adenocarcinoma of stomach and oesophagus (Chan et al., 2016), non-small cell lung cancer (Van Der Steen et al., 2016) and ovarian cancer (Moran-Jones, 2016). During carcinogenesis, mutations in the c-*MET* gene occur, enabling activation of the receptor even in the absence of a ligand. Another mechanism of dysfunction of the HGF pathway is autocrine stimulation and amplification of the c-*MET* gene (Lutterbach et al., 2007).

The role of c-MET-HGF pathway alterations in hepatocellular carcinoma has been comprehensively documented (Suzuki et al., 1994; Takayama et al., 1997) and has been proposed as a possible basis for targeted therapy of this tumour (Giordano and Columbano, 2014; Nakamoto, 2016; Pievsky and Pyrsopoulos, 2016). The studies showed that the role of the ligand (HGF) in the aberrant cellular signalling in HCC carcinogenesis is relatively less important than the role of the receptor itself. HGF is not overexpressed in HCC tissues and its transcription might be actually down-regulated (Tavian et al., 2000). The studies showed no gene number alterations of *HGF* either (Wang et al., 2013). On the other hand, it has been found that about 30 % of tumours evince overexpression of the c-MET protein (Lee et al., 2013); however, the biological basis for this augmentation in protein production could not be traced to point mutations (Guichard et al., 2012), and gene amplification was detected only in a small fraction of tumours (Takeo et al., 2001; Wang et al., 2013). It has been postulated that the biological basis for increased amounts of the c-MET protein in carcinomatous cells is an effect of hypoxia, leading to the activation of c-MET transcription (Pennacchietti et al., 2003).

Activation of the HGFR-c-MET pathway and overexpression of the c-MET protein have been associated with more aggressive behaviour of the neoplasm and poorer prognosis (Santoro et al., 2013; Trojan and Zeuzem, 2013), especially in terms of progression-free survival (Chu et al., 2013). The tumours with increased c-Met expression tended to be poorly or moderately differentiated (Suzuki et al., 1994).

Despite its well-documented and extensively researched role in hepatocellular cancer, the c-MET-HGF pathway was studied in hepatocellular adenoma only to a very limited degree. Studies in a mouse model suggested that cooperation between c-MET and transcription factor HNF-1α may play a role in development of this benign neoplasm; however, this mechanism was different from the one involving c-MET in tumorigenesis of HCC (Zucman-Rossi, 2008). A similar mouse model was employed to research the possible role of c-MET in development of hepatocellular adenomas induced by di(2-ethylhexyl) phthalate (DEHP), which acting through the PPARα receptor causes hypertrophy and hyperplasia of hepatocytes (Takashima et al., 2008). On the other hand, it has been shown that adenomas and carcinomas of the colon present a pattern of increasing c-MET expression from normal colon mucosa, through adenomas to colorectal cancer (Gayyed et al., 2015).

The aim of the present study was to assess the expression pattern and level of c-MET protein in hepatocellular adenoma (taking into account its status of the Wnt/β-catenin pathway functioning) and intertwining the results into a wider pattern of expression analysed by us in non-neoplastic liver and hepatocellular carcinoma of various histological grades.

Material and Methods

Ethics statement

The present study was approved by the Bioethics Committee of the 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) diagnosed with hepatocellular adenoma, 19 patients (14 males and 5 females, average age 52 years) diagnosed with hepatocellular carcinoma G3, 9 patients (6 males and 3 females, average age 62 years) diagnosed with hepatocellular carcinoma G1 or G2, and 11 patients (4 males and 7 females, average age 49 years) without any significant pathology of the liver. The histological grading of HCC was established according to the criteria listed in Table 1. 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.

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-c-MET 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, PA) in order to reduce unspecific binding of the antibody. The anti-Total c-MET (Ventana, Faulquemont, France) antibody or anti-β-catenin antibody (Dako, Glostrup, Denmark) was applied undiluted kept at room temperature for 30 min. Both antibodies used in the study were certified for *in vitro* diagnostics. In order to detect primary antibodies, secondary antibodies were used, directly conjugated with peroxidase particles catalogue number DS9800 (Leica Biosystems, Germany) 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-c-MET antibody were scanned in their entirety using a NanoZoomer-XR Digital slide scanner C12000 (Hamamatsu, Hamatsu City, Japan) at 40× 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 c-MET 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. Both, the presence and the intensity of staining were included into the analysis. A three-point scale was applied to diversify the intensity of immunoreaction, from high (3 points), through medium (2 points) to weak (1 point) value (see Table 2). The results were categorized by assigning a total score (enumerated as a sum of products of the medium percentage of immunopositive cells multiplied with the intensity value) and subjected to statistical analysis. Since the data obtained did not follow normal distribution (as assessed by Liljefors test), we used Kruskal-Wallis ANOVA and U-Mann-Whitney test to analyse differences between groups in terms of the c-MET expression score. P levels over 0.05 were deemed statistically significant.

Results

c-MET expression in hepatocellular adenoma as compared to non-neoplastic liver and hepatocellular carcinoma

The c-MET protein was present in the cell cytoplasm. We found that expression of the c-MET protein was highest in hepatocellular carcinomas of high histological grade (G3): median score – 1.58 (range 0.29–2.80, see Fig. 1d). This result was visibly higher than that observed across other groups. For hepatocellular carcinomas of low or medium histological grade (G1 or G2), the median amounted to 0.48 (range 0.24–0.85, see Fig. 1c), for non-neoplastic liver the median c-MET score was 0.58 (0.13–1.14). For hepatocellular adenoma the median was 0.63 (0.22–2.78, see Fig. 2). The differences between groups were statistically significant at P = 0.0134, H = 10.7.

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

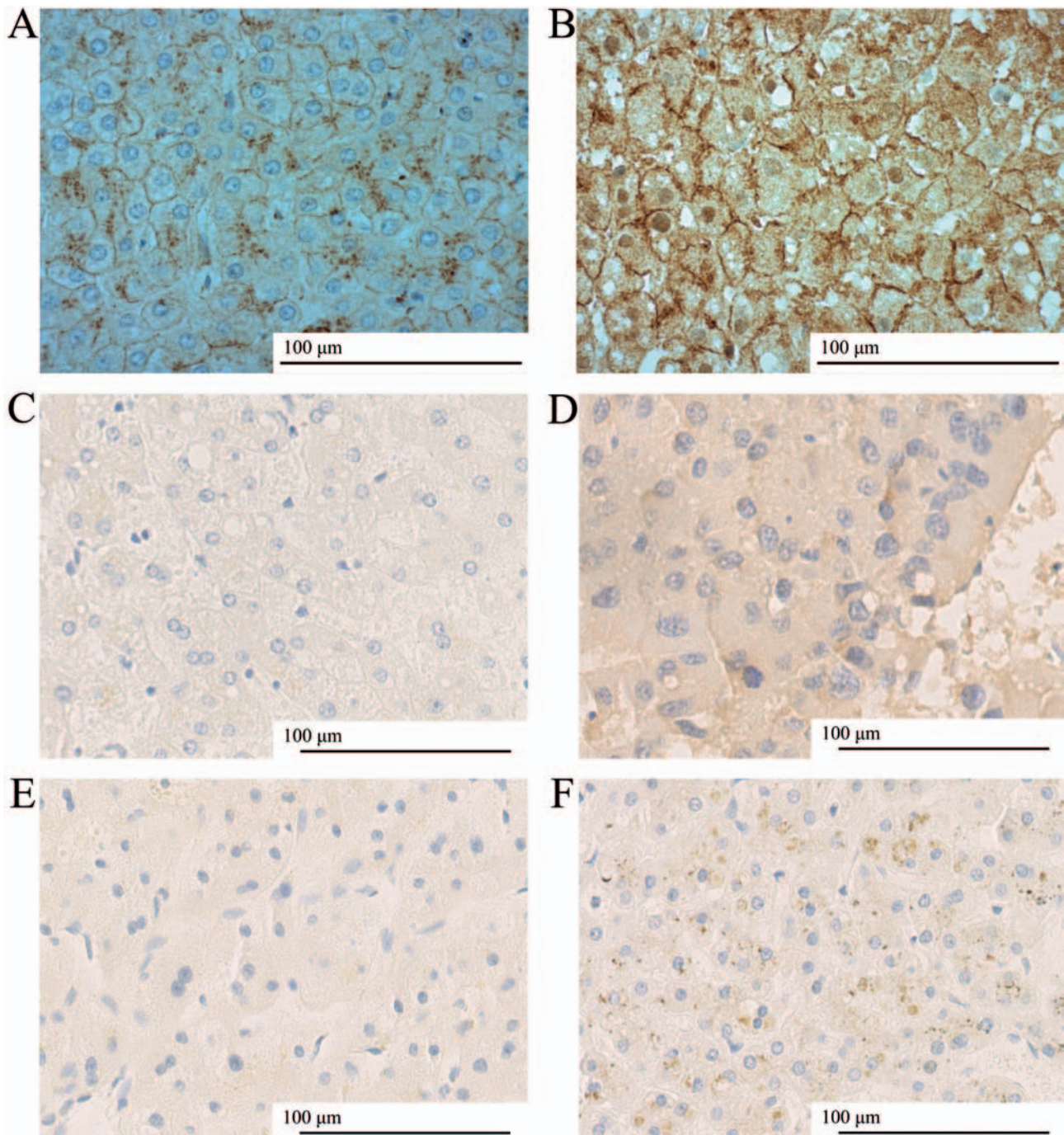| Grade	Microscopic features
G1	Small tumour cells, resembling normal liver in their shape and trabecular arrangement
G2	Small degree of nuclear irregularity, hyperchromatism
G3	Prominent nuclear pleomorphism, marked hyperchromatism, anaplastic cells

Fig. 1. Representative slides demonstrating immunohistochemical reactions (40x magnification). Hepatocellular adenoma without (**A**) and with nuclear expression of β-catenin (**B**). Immunohistochemical staining with anti-c-MET antibody – cytoplasmic reaction (C-F): well-differentiated hepatocellular carcinoma (**C**, score 0.3), poorly-differentiated hepatocellular carcinoma (**D**, score 2.1), hepatocellular adenoma with (**E**, score 0.15) and without (**F**, score 1.8) β-catenin pathway dysfunction.

Table 2. The scale for semi-quantitative analysis of the intensity of immunuhistochemical reaction

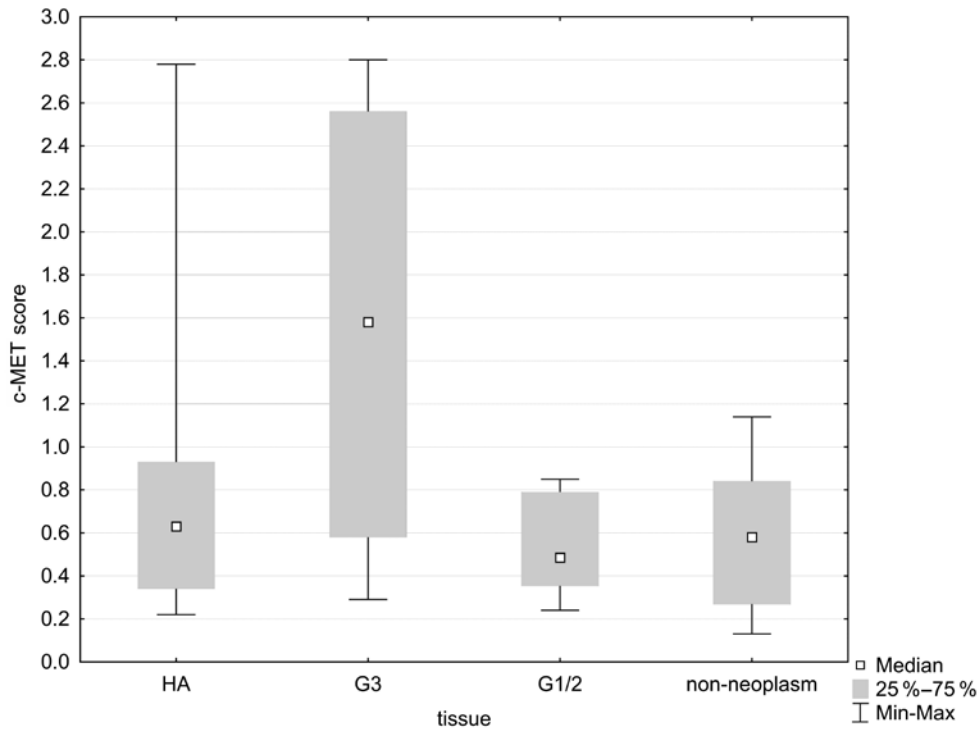| Score	Description
0	No immunohistochemical reaction present
1	Weak immunohistochemical reaction, present only focally in the cells
2	Moderate immunohistochemical reaction, weak intensity in the whole cytoplasm or strong focal cytoplasmic reaction
3	Strong immunohistochemical reaction, strong intensity in the whole cytoplasm

Fig. 2. Comparison of c-MET expression in non-neoplastic and neoplastic liver tissues
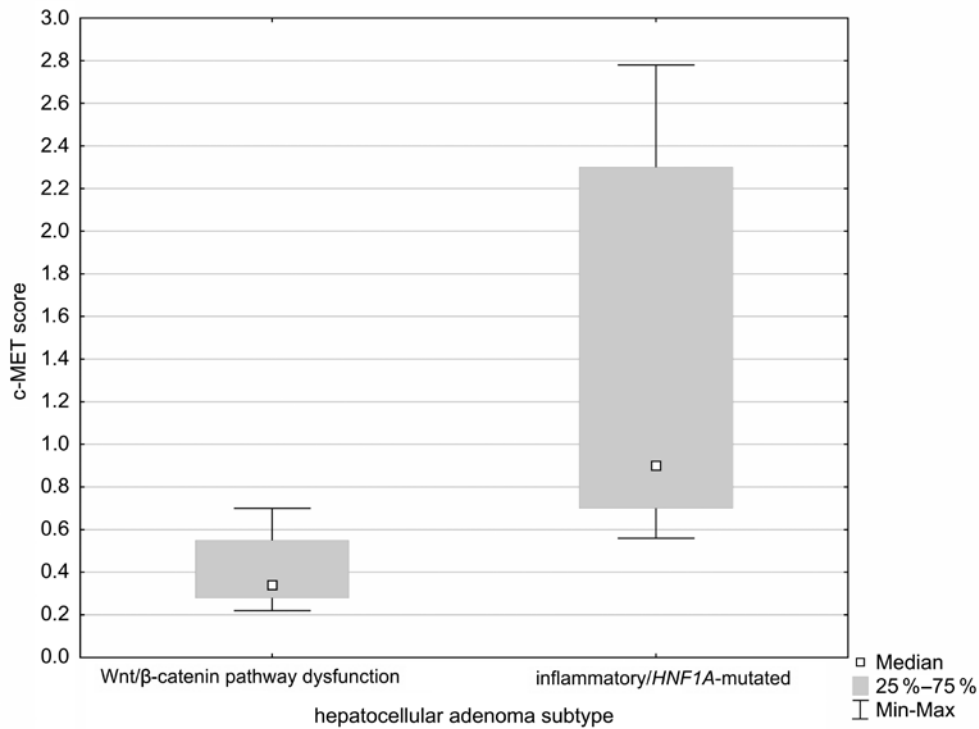

Fig. 3. Comparison of c-MET expression in hepatocellular adenoma subtypes

c-MET expression in hepatocellular adenomas divided on the basis of β-catenin status

Hepatocellular adenomas with nuclear overexpression of β-catenin had significantly lower expression of the c-MET protein in terms of score calculated by us (median 0.34, range 0.22–0.70) compared to adenomas without overexpression of β-catenin (median 0.90, range 0.56–2.78). The results were statistically significant at $P = 0.000152$, $Z = 3.35$ (see Fig. 1 e,f and Fig. 3).

Discussion

In the present study we found that expression of c-MET might be strongly increased in neoplastic cells originating from hepatocytes, both in malignant and benign tumours. Our findings on the one hand confirmed the previously published results (Suzuki et al., 1994) that associated c-MET overexpression with worse differentiation of HCC cells. On the other hand, we found that low- and moderate-grade HCCs did not differ significantly in terms of c-MET expression from non-neoplastic liver cells. What is most important and novel is the position of HAs between these two groups of malignant tumours in terms of c-MET expression. The median expression score in this group was only slightly higher than that observed in HCC G1/G2; however, as can be seen in Fig. 1, this group was very heterogeneous as far as the expression of c-MET is concerned.

Many cases evinced results comparable to those of HCCs G3, and far above those of any case in the non-neoplastic liver or HCC G1/G2 group. This heterogeneity might be easily explained with reference to the division of HAs into three groups of different pathogenic background, as described in Introduction. Especially the Wnt/β-catenin dysfunction-associated type seems to be important, when an attempt to explain neoplastic transformation of liver cells is made, due to the observation of frequent β-catenin overexpression in the nuclei of malignant tumour cells (Chen et al., 2013; Yong et al., 2016).

We explored this possibility further by dividing the group of HAs into subgroups based on the status of β-catenin overexpression in the nucleus. These two groups were indeed significantly different as far as c-MET expression is concerned; however, the relation seems to be reverse, that is HAs overexpressing β-catenin in the nucleus were characterised by lower levels of c-MET expression.

This finding indeed provides a basis for the heterogeneity observed when analysing the group of HAs as a whole. The clear separation of the two groups based on c-MET expression (the ranges of c-MET scores obtained from both groups were hardly overlapping) showed that Wnt/β-catenin dysfunction-associated type of HA expresses c-MET in similar amounts to non-neoplastic liver and well- to moderately-differentiated HCC. In contrast to this group, HAs without nuclear β-catenin overexpression (presumably belonging to the inflammatory or *HNF1A* mutation-associated type) presented a similar pattern of c-MET expression to poorly-differentiated HCC.

This pattern of differences in c-MET expression between HA subtypes might seem surprising and contrary to what has been observed in adenomas in other locations with known propensity to malignant transformation, such as colon adenomas. In these tumours, the accumulation of mutations begins with Wnt/β-catenin pathway dysfunction (through *APC* mutation), which is an early event in the adenoma-carcinoma sequence (Armaghany et al., 2012). Parallel to this development, it has been recently found that c-MET expression rises progressively from normal colon mucosa through adenoma to carcinoma (Gayyed et al., 2015). However, earlier studies suggest that reduction of HGF expression with no concomitant increase in c-MET expression might be an important event in malignant transformation of colon adenomas (Trovato et al., 2004), so this matter is far from cleared.

It has been noted that both c-MET overexpression and Wnt/β-catenin pathway dysfunction are common features of a number of malignant tumours. Furthermore, among HAs, Wnt/β-catenin pathway dysfunction has been identified as one of the markers of increased risk for malignant transformation (Van der Borght et al., 2007). However, it should be stressed that not only this type of HA could be a background for HCC development (Monga, 2006). The matter is further addled by the recent observation that immunohistochemistry is not able to precisely identify HA subtypes, as verified by DNA sequencing (Margolskee et al., 2016).

On the other hand, it should not amaze that in our study the subgroup of HAs evincing nuclear overexpression of β-catenin appeared more similar to well- and moderately-differentiated HCCs as far as c-MET expression is concerned. Although large systematic reviews and meta-analyses of malignant transformation of HAs in terms of grading or resulting HCC are lacking, it might be cogently guessed (and confirmed by case-reports (Larson et al., 2002) and studies involving single populations (An et al., 2015)) that in case of malignancy occurring within HA, the resultant carcinoma should histologically resemble the benign tumour, that is should be well-differentiated. We postulate that HAs of the Wnt/β-catenin pathway dysfunction type might progress into well-differentiated HCCs without any significant change in c-MET expression, through other, not yet identified mutations or gene-transduction alterations. The further progression in terms of histological dedifferentiation might be contributed to by overexpression of c-MET. Therefore, it could be supposed that during malignant transformation and further development of malignant tumour, amplification of the c-*MET* gene and other alterations leading to overexpression of the c-MET protein are rather late events. This mechanism and order of genetic disruptions is different from what has been observed in the adenoma-carcinoma sequence of colorectal carcinoma (see above).

In contrast, HAs that have been identified as belonging to the inflammatory or *HNF1A* mutation-associated type overexpress c-MET protein as a part of multiple, not yet pin-pointed spectrum of molecular changes causing tumour progression, however without tendency to malignant transformation. The indolent course of these neoplasms proves that c-MET overexpression is not enough to pose a risk for malignancy in the absence of Wnt/β-catenin pathway changes. These findings might suggest that two independent mechanisms of c-MET overexpression exist. One could cause early occurrence

of protein accumulation in HAs not tending to develop into carcinomas, the other could be observed as a late event in malignant tumour progression. Another explanation is that the constellation of genetic and molecular factors plays a decisive role in the biological and clinical outcome of c-MET overexpression present. When this takes place in the benign setting of HAs with inflammation or *HNF1A* mutation, no further danger to the patient ensues, whereas its appearance in already malignant, yet still well-differentiated tumours, might be interpreted as a step towards worse outcome and prognosis associated with poor differentiation of tumour cells. These connections should be further studied by delving deeper into the genetic basis for c-MET overexpression observed in the present study, for example by analysing mutations in the c-*MET* gene and gene amplification.

Conclusions

In conclusion, expression of the c-MET protein in hepatocellular adenoma is variable and differs between molecular subtypes of this neoplasm: inflammatory and *HNF1A* mutation-associated types are characterized by overexpression of c-MET to an extent comparable with poorly-differentiated HCC, whereas the Wnt/β-catenin dysfunction-associated type lacks overexpression, and the amount of c-MET protein accumulated in its cells is similar to the levels in non-neoplastic tissue and well- to moderately-differentiated HCC.

These findings suggest that the molecular changes leading to c-MET overexpression are a late event in hepatocarcinogenesis and occur at more advanced stages during progression within the carcinomatous tumour itself. Unfortunately, this finding precludes the possibility of c-MET staining to be used diagnostically to differentiate between well-differentiated HCC and HA. This usage could be made more difficult due to the heterogeneity of the latter group as well. Nonetheless, placing c-MET overexpression in the wider context of malignant and non-neoplastic lesions (as has been attempted in the present study) is a step towards recreating the chronological sequence of molecular disruptions materialising during the progression of neoplastic changes in hepatocytes. Further studies will be needed to evaluate the underlying mechanism of c-MET overexpression in HA and HCC more precisely, and similar studies could be of use for other proteins involved in the malignant transformation of hepatocytes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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