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Characteristic of expression levels of HepPar-1, alpha-fetoprotein, cytokeratin 7 and 20 by the cells of cholangiocellular cancer in trephine biopsy of the liver Zaporozhye State Medical University

Key words: Cholangiocellular Carcinoma, Expression, HepPar 1, alpha-Fetoprotein (AFP), Cytokeratin 7, Cytokeratin 20.

Aim. Expression level of immunohistochemical markers such as HepPar-1, AFP, CK7, CK20 and the area of immunopositive cells in cholangiocellular liver cancer, and their differences from hepatocellular carcinoma were investigated.

Methods and results. Histopathological, histochemical and immunohistochemical research of trephine was determined in the liver in 90 patients with biopsy. Among them 53 patients had hepatocellular, 36 – cholangiocellular liver cancer, 1 patient had mixed hepato-cholangiocellular carcinoma. Level of expression of immunohistochemical markers of tumor cells and the area of immunopositive tumor cells in the tumor was determined by photo-digital morphometry. It was established that expression of α -fetoprotein is determined in 47.22% of patients with cholangiocellular liver carcinoma in tumor cells, when AFP-immunopositive cells represent $17,25 \pm 9,67\%$ of the total area of tumor cells. Positive expression of HepPar-1 cells in cholangiocellular carcinoma wasn't detected (unlike hepatocellular carcinoma, when cytoplasmic expression of HepPar-1 by tumor hepatocytes is determined in 92.45% of cases). Expression of CK7 by cholangiocellular carcinoma cells was observed in 97.22% of patients, and the expression of CK20 – in 45.29% patients, immunopositive cells represent $43,55 \pm 9,93\%$ and $50,28 \pm 16,35\%$ of the tumor area, respectively. Medium strength correlation was determined between the level of AFP and CK7 expression by tumor cells in cholangiocellular carcinoma. Direct strong bond was determined between level of AFP and CK20 expression. Negative weak correlation was determined between the level of CK7 and CK20.

Характеристика рівня експресії НерPar-1, альфа-фетопротеїну, цитокератинів 7 і 20 клітинами холангіоцелюлярного раку у трепанобіоптатах печінки

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З метою визначення рівня експресії імуногістохімічних маркерів НерPar-1, AFP, CK7, CK20, площі імунопозитивних клітин у холангіоцелюлярному раку печінки та їхніх відмінностей від гепатоцелюлярного раку здійснили патогістологічне, гістохімічне й імуногістохімічне дослідження трепанобіоптатів печінки 90 хворих, серед них 53 особи із гепатоцелюлярним, 36 – холангіоцелюлярним раком печінки, у 1 хворого діагностували змішану гепато-холангіоцелюлярну карциному. Рівень експресії імуногістохімічних маркерів пухлинними клітинами і площу імунопозитивних клітин у пухлині визначали за допомогою фотоцифрової морфометрії. Встановили, що у 47,22% хворих на холангіоцелюлярний рак печінки в пухлинних клітинах визначається експресія α -фетопротеїну, AFP-імунопозитивні клітини становлять $17,25 \pm 9,67\%$ загальної площі клітин пухлини. Не виявили позитивної експресії НерPar-1 клітинами холангіоцелюлярного раку печінки (на відміну від гепатоцелюлярної карциноми, в якій у 92,45% випадків визначається цитоплазматична експресія НерPar-1 пухлинними гепатоцитами). Експресію СК7 клітинами холангіоцелюлярної карциноми відзначили у 97,22% хворих, експресію СК20 – у 45,29% хворих, імунопозитивні клітини становлять $43,55 \pm 9,93\%$ і $50,28 \pm 16,35\%$ площі пухлині відповідно. У холангіоцелюлярній карциномі між рівнями експресії пухлинними клітинами AFP і СК7 виявили пряму середньої сили кореляцію, між рівнями експресії AFP і СК20 – прямий сильний зв'язок, між рівнем експресії СК7 і СК20 – негативну слабкої сили кореляцію.

Ключові слова: холангіоцелюлярний рак, експресія, Нер Par 1, α -фетопротеїн (AFP), цитокератин 7, цитокератин 20.

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Характеристика урвня експресии НерPar-1, альфа-фетопротеина, цитокератинов 7 и 20 клетками холангиоцеллюлярного рака в трепанобиоптатах печени

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С целью определения уровня экспрессии иммуногистохимических маркеров НерPar-1, AFP, CK7, CK20, площади иммунопозитивных клеток в холангиоцеллюлярном раке печени и их отличий от гепатоцеллюлярного рака проведено патогистологическое, гистохимическое и иммуногистохимическое исследование трепанобиоптатов печени 90 больных, среди которых 53 пациентов страдали гепатоцеллюлярным, 36 – холангиоцеллюлярным раком печени, у 1 больного диагностирована смешанная гепато-холангиоцеллюлярная карцинома. Уровень экспрессии иммуногистохимических маркеров опухолевыми клетками и площадь иммунопозитивных опухолевых клеток в опухоли определяли фотоцифровой морфометрией. Установили, что у 47,22% больных холангиоцеллюлярной карциномой печени в опухолевых клетках определяется экспрессия α -фетопротеина, AFP-иммунопозитивные клетки составляют $17,25 \pm 9,67\%$ общей площади клеток опухоли. Не обнаружили положительной экспрессии НерPar-1 клетками холангиоцеллюлярного рака печени (в отличие от гепатоцеллюлярной карциномы, в которой в 92,45% случаев определяется цитоплазматическая экспрессия НерPar-1 опухолевыми гепатоцитами). Экспрессия СК7 клетками холангиоцеллюлярной карциномы установлена у 97,22% больных, экспрессия СК20 – у 45,29% больных, иммунопозитивные клетки составляют $43,55 \pm 9,93\%$ и $50,28 \pm 16,35\%$ площади опухоли соответственно. В холангиоцеллюлярной карциноме между уровнем экспрессии опухолевыми клетками AFP и СК7 отмечена прямая средней силы корреляция, между уровнем экспрессии AFP и СК20 – прямая сильная связь, а между уровнем экспрессии СК7 и СК20 – отрицательная слабой силы корреляция.

Ключевые слова: холангиоцеллюлярный рак, экспрессия, НерPar-1, α -фетопротеин (AFP), цитокератин 7, цитокератин 20.

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In last years there is a trend towards to increasing of primary liver cancer in the world, which includes hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) among adults, and hepatoblastoma among children. In the United States, in the structure of primary cancers of the hepatobiliary system hepatocellular carcinoma accounts for 80%, cholangiocarcinoma - 10-20%; while among its subtypes cholangiocellular carcinoma (CC) is 40%, cholangiocarcinoma porta hepatis (Klatskin tumor) - 7%, extrahepatic (peripheral) cholangiocarcinoma - 53% [1].

For the differential pathomorphological diagnosis of hepato- and cholangiocellular liver cancer the minimum immunohistochemical panel is recommended, which is used for determining of the expression of hepatocyte specific antigen by tumor cells (HepPar 1), α -fetoprotein (AFP), polyclonal carcinoembryonic antigen (rCEA) mucikarmin, cytokeratins (CK7, CK8, CK18, CK19, CK20), in scientific researches, additionally, fetal liver expression of proteoglycan is determined (glypican-3), Factor XIIIa, alpha-1-antitrypsin deficiency, thyroid transcription factor-1 (TTF-1), common antigen of acute lymphoblastic leukemia (CD10), CD56 (NCAM), claudin, villin and mucin (MUC1, MUC2, MUC4) [2- 4].

Immunohistochemical (IHC) researches with trephine biopsy puncture of the liver acquired special differential diagnostic importance, because of the containing of a limited amount of diagnostic material. Without them, it is almost impossible to identify hepato- and cholangiocellular phenotype of solidcellular and low-grade cancer, differential diagnosis is impossible when a trephine biopsy doesn't contain the characteristic patterns of hepatocellular carcinoma or tubular pattern of cholangiocellular cancer. Contradictory results of extended researches of last years, outlined in modern manuals [2,3,5], have shown a great variability of expression levels of HepPar-1, AFP, CK7, 8, 18, 19 and 20 by tumor cells of hepato- and cholangiocellular cancer, so this problem needs to be further developed.

Aim of the study

To determine level of expression of immunohistochemical markers HepPar-1, AFP, CK7, CK20 and area of immunopositive cells in cholangiocellular carcinoma of the liver and their differences from hepatocellular carcinoma.

Materials and methods

A complex histopathological, histochemical and immunohistochemical (IHC) methods was used in 90 patients. 53 (58.9%) patients had HCC and 36 (40%) – CC liver, in 1 patient (1.1%) mixed hepato-cholangiocellular carcinoma was found. The average age of patients with HCC was 59,6 \pm 11,32 years (26–73 years), HTSK – 58,87 \pm 11,72 years (33–83 years). In the control group only 5 patients with somatic diseases without clinical, biochemical and morphological signs of liver damage undergone liver biopsies.

Columns of trephine liver biopsies from patients with HCC and CCK were fixed in 10% buffered formalin and embedded in paraffin. With help of rotary microtome HM-3600 (MICROM Laborgerate GmbH - Germany) serial 3-4 micron thick sections were made for staining with hematoxylin and eosin, Van Gison and Masson-tricolor and for IHC studies.

Liver tissue in paraffin sections undergone IHC study in accordance with standardized protocols after the temperature antigen unmasking and suppression of endogenous peroxidase activity. Primary antibodies and visualization system DAKO EnVision + System («DAKO», Denmark) with diaminobenzidine (DAB) were used. Monoclonal antibodies CK7 were used (Ks20.8 clone) to determine the cytokeratin profile of HCC and CCK cells. Polyclonal antibodies CK20, cells of CCK and HCC were marked with help of monoclonal antibodies Hepatocyte Specific Antigen (HepPar-1 clone OCH1E5) and polyclonal antibodies against α -fetoprotein (AFP) (all reagents are «DAKO», Denmark).

The level of expression of immunohistochemical markers by tumor cells and the area of immunopositive tumor cells in the tumor were determined by photo digital morphometry. To quantify the expression level of HepPar-1, AFP, CK7 and CK20 in each observation of liver cancer, micropreparates with the corresponding immunopositive reaction were photographed by digital camera «Olympus 3040» (Japan) in the Axioplan 2 microscope («Carl Zeiss», Germany) with an increasing of x200 in 5 fields of view and subsequently analyzed using medical program of digital image processing Image J [Rasband WS (1997–2012)]. Level of expression of the relevant immunohistochemical markers was determined in the plug Colour Deconvolution of this program. According to the standard brightness scale the view was graded quantitatively by A. Katayama et al. (2004) in points (from 0 – white to 255 – black) and was divided into 4 categories: negative reaction – 0–20 points; low level of expression – 21–50 points; moderate level of expression – 51–100 points; high level of expression – over 100 points.

Program Image J was used for morphometric measurements of the area, which is occupied by Hep Par-1, AFP-, CK7- and CK 20-immunopositive cells in the digital images of immunohistochemical hepato- and cholangiocellular liver cancer. Total area of the expression of each listed marker was determined, which was represented as a percentage ratio of immunopositive pixels number of corresponding marker to the total number of pixels in the image, expressed in %.

Statistical processing of the results was performed on a personal computer using program «STATISTICA® for Windows 6.0» (StatSoft Inc., License № AXXR712D833214FAN5). The average value (M), standard deviation (σ), the standard error of the representativeness of the mean value (m) were calculated, also the 95% confidence interval of the mean was calculated as well. Correlation was identified by calculating of the Pearson's coefficient (for nonparametric data). Results were considered as significant at p < 0.05.

Results and discussion

Immunohistochemical studies results showed that cytoplasmic and nuclear expression of α -fetoprotein was determined in malignant cells in 81.13% of patients with HCC and 47.22% of patients with CC liver cancer. Alpha-fetoprotein is carcinoembryonic protein and is produced in the liver and internal organs of the yolk sac endoderm, as well as in the cells of malignant tumors of the liver [6]. It was found that the level of AFP expres-



sion by tumor cells and area of the AFP-immunopositive cells in the CC is lower than in HCC of the liver. During analyzing of the expression of AFP by CC cells it was found that 27.78% of patients had moderate expression level of this glycoprotein and was $68,51 \pm 15,09$ points, in 19.44% of patients it was low ($34,62 \pm 10,51$ points), 52.78% of patients had negative level of the AFP expression by CC cells (expression level was $6,51 \pm 2,87$ points).

With help of digital photo morphometry we found that the area occupied by AFP-immunopositive cells was $17,25 \pm 9,67\%$ of the total area of cholangiocellular cancer cells. According to S.A. Geller, L.M. Petrovic et al. [2] data no more than 10% of CC liver are AFP-immunopositive; most part of the pathologists notice that AFP is marker of hepatocellular liver differentiation of malignant cells, however, the sensitivity of this marker in the FCC, according to different authors, vary from 15% to 70% [2,7].

HepPar-1 expression (Hepatocytes paraffin-1) normally reacts with hepatocytes mitochondrial enzymes, renal tubular epithelium and intestinal epithelium [8]. Positive expression of HepPar-1 by cells in cholangiocellular liver cancer wasn't detected. Positive cytoplasmic expression of HepPar-1 by tumor cells was detected in 92.45% of patients with hepatocellular carcinoma [9], as well as in the tubular component of mixed hepato-cholangiocellular carcinoma of the liver. Our results are consistent with results of other researchers. According to S.A. Geller, L.M. Petrovic et al. [2] and A. Lugli et al. [10] all cholangiocarcinomas are HepPar-1-immunonegative, while HepPar-1 expression is detected in 80-90% of hepatocellular carcinomas [1,2], which indicates that expression of HepPar-1 is represented in some CC.

At the basis of using antibodies against cytokeratins for differential immunohistochemical diagnosis of primary liver cancer in adults is the idea that the most likely source of hepatocellular, cholangiocellular and mixed hepatocellular-cholangiocellular carcinoma are hepatic progenitor cells localized in the tubules Goering, which normally differentiate into hepatocytes and cholangiocytes [4,11]. Consequently, tumor hepatocytes (as normal) retain expression of low molecular cytokeratins 8 and 18 and cholangiocellular cancer cells (like cholangiocytes as well biliary ducts as cholangioles) characterized by expression of cytokeratins 7, 19 and 20 [4].

Study of the expression of CK7 and CK20 by CC cells showed the following. Positive cytoplasmic expression of CK7 by tumor cells was detected in 97.22% of cholangiocellular carcinomas. At the same time in 54.72% of the cases of CC, the high level of CK7 expression was determined ($114,03 \pm 11,53$ points), in 31.17% of the patients moderate level of expression was found ($71,06 \pm 14,68$ points), in 8.55% weak level of CK7 expression by tumor cells was found ($43,32 \pm 6,71$ points), and in 2.78% of patients CK7 expression in the tumor was negative (detection limit was $10,99 \pm 3,71$ points). Cytokeratin-7-positive cells were unevenly distributed in malignant cholangiocellular tumors, the average size of CK7-immunopositive cells in CCK was $43,55 \pm 9,93\%$. In accordance with our previously published results CK7 expression was also detected in 37.74% of HCC cases.

According to information published by David J. Dabbs in manual of diagnostic immunohistochemistry, the fetal hepatocytes contain cytokeratins CK8, 18 and 19; then after 10-th week of gestation, they lose CK19, so mature hepatocytes express only CK8 and CK18. Cholangiocytes of intrahepatic bile ducts in the adult patients liver express CK7,8,18,19, and often are CK20-immunonegative [3]. Expression of CK7 by CC tumor cells is detected in the majority of CC and it is usually weakly positive or negative in HCC [12], that is why CK7 considered as the most appropriate marker of CC.

Our immunohistochemical researches shown that positive cytoplasmic expression of CK20 by CC tumor cells occurs in 45.29% of patients. High expression level of CK20 ($115,15 \pm 13,69$ points) was observed in 24.27% of cases, in 11.17% of the patients moderate expression level of cytokeratin was found ($81,76 \pm 16,48$ points), in 9.85% of patients the CK20 expression in CC was weak ($33,32 \pm 7,61$ points), in 54.08% of patients the CK20 expression in CC was negative (only $8,51 \pm 2,89$ points). Area of CK20-immunopositive cells in CC was $50,28 \pm 16,35\%$. Expression of CK20 by HCC cells was determined rarer – in 30.13% of patients [9].

Normally, CK20-positive cells were localized in the gastric and intestinal epithelium, urothelium and Merkel cells of the skin appendages. Despite the fact that CK20 seemed like an intestinal epithelium marker, it is found in 20% of malignant liver tumors [3]. According to A.D. Burt et al. data [1] presence of CK20-immunopositive cells in liver malignant tumors is used for the differential diagnosis between primary hepato, cholangiocellular carcinoma of the liver and liver metastasis in colorectal cancer, stomach or other organs.

Expression levels of AFP, CK7, CK20, HepPar-1 and correlations between them in CCK are shown in table 1.

Table 1

The expression levels of AFP, CK7, CK20, HepPar-1 and correlations between them in cholangiocellular liver cancer

Characteristic of expression level	Cholangiocellular carcinoma			
	AFP (A)	CK7 (B)	CK20 (C)	Hep Par 1 (D)
The average level of expression %	37,88±25,23	40,73±25,23	50,28±16,35	0
p	<0,05			
r _{A-B}		0,5		
r _{A-C}			1	
r _{B-C}		-0,1		

Correlation analysis showed negative, weak force correlation (the Pearson coefficient $r = -0,1$) between expression level of CK7 and CK20 in the tumor cells in patients with CC. There was direct correlation of medium strength (the Pearson coefficient $r = +0,5$) between level of AFP and CK7 expression. And direct strong bond (the Pearson coefficient $r = +1$) between level of AFP and CK20 expression.



Results

1. Expression of α -fetoprotein by tumor cells was determined in 47.22% of patients with cholangiocellular liver carcinoma, AFP-immunopositive cells constituted $17,25 \pm 9,67\%$ of the total area of tumor cells.

2. Expression of HepPar-1 cholangiocellular carcinoma cells was not detected (unlike hepatocellular carcinoma, cytoplasmic expression of HepPar-1 was determined in 92.45% of the tumor hepatocytes).

3. 97.22% of the patients with cholangiocellular carcinoma expressed cytoplasmic CK7 in tumor cells, immunopositive cells constituted $43,55 \pm 9,93\%$ of carcinoma area.

4. Expression of cytoplasmic CK20 was determined in cells of 45.29% of patients with cholangiocellular carcinoma, immunopositive cells constituted $50,28 \pm 16,35\%$ of the tumor area.

5. Weak force correlation (the Pearson coefficient $r = -0,1$) was founded between expression level of CK7 and CK20 in the

tumor cells in patients with CC. There was direct correlation of medium strength (the Pearson coefficient $r = +0,5$) between level of AFP and CK7 expression. And direct strong bond (the Pearson coefficient $r = +1$) between level of AFP and CK20 expression.

Conclusion

The main immunohistochemical difference between cholangiocellular carcinoma and hepatocellular carcinoma is the lack of HepPar-1 expression by its cells, and also more than 2-fold lower level of expression of α -fetoprotein. Variable level of expression of cytokeratins in cholangiocellular and hepatocellular carcinoma is likely due to the presence of various impurities clones with abnormal cholangiosimilar and hepatosimilar cellular differentiation. The resulting comparative data should be considered in the differential diagnosis of immunohistochemical cholangiocellular and hepatocellular carcinoma in trephine biopsy of the liver with limited diagnostic material.

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