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

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**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 hepatocolo-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 ± 9.67% of the total area of tumor cells. Positive expression of HepPar-1 cells in cholangiocellular liver cancer 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 ± 9.93% and 50.28 ± 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.

Characteristica rівня експресії HepPar-1, альфа-фетопротеїну, цитокератинів 7 і 20 клітинами холангиоцеллюлярного раку у трепанобіоптатах печінки

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З метою визначення рівня експресії імуногістохімічних маркерів HepPar-1, AFP, CK7, CK20, площі імунопозитивних клітин у холангиоцеллюлярному раку печінки та їхніх відмінностей від гепатоцеллюлярного раку здійснено патогістохімічне, гістохімічне й імуногістохімічне дослідження трепанобіоптатів печінки 90 хворих, серед яких 53 особи із гепатоцеллюлярним, 36 – холангиоцелюлярним раком печінки, у 1 хворого діагностували змішану гепато-холангиоцеллюлярну карциному. Рівень експресії імуногістохімічних маркерів пухлинними клітинами і площу імунопозитивних клітин у пухлині визначали за допомогою фотоцифрової морфометрії. Встановлено, що у 47,22% хворих на холангиоцеллюлярний рак печінки в пухлинних клітинах визначається експресія α-фетопротеїну, AFP-імунопозитивні клітини становлять 17,25±9,67% загальної площі клітин пухлини. Не виявили позитивної експресії HepPar-1 клітинами холангиоцеллюлярного раку печінки (на відміну від гепатоцеллюлярної карциноми, в якій у 92,45% випадків визначається цитоплазматична експресія HepPar-1 пухлинними гепатоцитами). Експресію CK7 клітинами холангиоцеллюлярної карциноми відзначали у 97,22% хворих, експресію CK20 – у 45,29% хворих, імунопозитивні клітини становлять 43,55±9,93% і 50,28±16,35% площі пухлини відповідно. У холангиоцеллюлярній карциномі між рівнями експресії пухлинними клітинами AFP і CK7 виявили пряму середньої сили кореляцію, між рівнями експресії АFP і CK20 – прямий сильний зв’язок, між рівнем експресії CK7 і CK20 – негативну слабку сили кореляцію.

**Ключові слова:** холангиоцеллюлярний рак, експресія, Hep Par 1, α-фетопротеїн (AFP), цитокератин 7, цитокератин 20.

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Характеристика уровня экспрессии HepPar-1, альфа-фетопroteина, цитокератинов 7 и 20 клетками холангиоцеллюлярного рака в трепанобиопсатах печени

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С целью определения уровня экспрессии иммуногистохимических маркеров HepPar-1, AFP, CK7, CK20, площади иммунопозитивных клеток в холангиоцеллюлярном раке печени и их отличий от гепатоцеллюлярного рака проведено патогистологическое, гистохимическое и иммуногистохимическое исследование трепанобиопсата 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. Установлено, что у 47,22% больных холангиоцеллюлярной карциномой печени в опухолевых клетках определяется экспрессия а-фетопротеина, AFP-иммунопозитивные клетки составляют 17,25±9,67% общей площади клеток опухоли. Не обнаружили положительной экспрессии HepPar-1 клетками холангиоцеллюлярного рака печени (в отличие от гепатоцеллюлярной карциномы, в которой в 92,45% случаев определяется цитоплазматическая экспрессия HepPar-1 опухолевыми гепатоцитами). Экспрессия CK7 клетками холангиоцеллюлярной карциномы установлена у 97,22% больных, экспрессия CK20 – у 45,29% больных, иммунопозитивные клетки составляют 43,55±9,93% и 50,28±16,35% площади опухоли соответственно. В холангиоцеллюлярной карциноме между уровнем экспрессии опухолевыми клетками AFP и CK7 отмечена прямая средней силы корреляция, между уровнем экспрессии AFP и CK20 – прямая сильная связь, а между уровнем экспрессии CK7 и CK20 – отрицательная слабой силы корреляция.

**Ключевые слова:** холангиоцеллюлярный рак, экспрессия, HepPar-1, а-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 hepatocellular cholangiocarcinoma liver cancer the minimum immunohistochecmical 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 (CEA), mucikarin, 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 hepatocellular cholangiocarcinoma 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 hepatocellular cholangiocarcinoma, 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 hepatocellular cholangiocarcinoma was found. The average age of patients with HCC was 59.6±11.32 years (26–73 years), HTSK – 58.87±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 biopsts 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 (Rashb W 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 CK20-immunopositive cells in the digital images of immunohistochemical hepatocellular 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 № AXXR712D833214FANS). 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-
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_{as}                            | 0.5                         |                          |                          |              |
| r_{sc}                            | 1                           |                          |                          |              |
| r_{sc}                            | -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 ± 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 was found cytoplasmic CK7 in tumor cells, immunopositive cells constituted 43.55 ± 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 ± 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 CK20 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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