

Chromogenic *In Situ* Hybridization Analysis of Epidermal Growth Factor Receptor Gene/Chromosome 7 Numerical Aberrations in Hepatocellular Carcinoma Based on Tissue Microarrays

Evangelos Tsiambas · Loukas Manaios ·
Costas Papanikolopoulos · Dimitrios N. Rigopoulos ·
Dimitrios Tsounis · Andreas Karameris ·
Aspasia Soultati · Antigoni Koliopoulou ·
Christos Kravvaritis · Theodoros Sergentanis ·
Efstratios Patsouris · Spyridon Dourakis

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Abstract Although Epidermal Growth Factor Receptor (EGFR) overexpression is observed frequently in hepatocellular carcinomas (HCC), specific gene deregulation mechanisms remain unknown. Our aim was to investigate the prognostic significance of the combined protein and gene/chromosome 7 numerical alterations. Using tissue microarray technology, thirty-five ($n=35$) paraffin embedded histologically confirmed HCCs were cored and re-embedded in a paraffin block. Immunohistochemistry was performed for the determination of EGFR protein levels and evaluated by the performance of digital image analysis.

Chromogenic *in situ* hybridization was also performed based on the use of EGFR gene and chromosome 7 centromeric probes, respectively. EGFR overexpression was observed in 26/35 (74.2%) cases and was correlated to the grade of the tumors and also to the history of the patients ($p=0.013$, $p=0.036$, respectively). Numerical alterations regarding gene and chromosome 7 were identified in 4/35 (11.4%) and 12/35 (43.2%) cases associated to the grade of the tumors ($p=0.019$, $p=0.001$, respectively) and to the survival rate of the patients ($p=0.037$, $p=0.001$, respectively). EGFR overall expression was also correlated

E. Tsiambas
Dept of Molecular Cytopathology, 417 NIMTS Hospital,
Athens, Greece

L. Manaios
Dept of Surgery, Bioclinic,
Athens, Greece

C. Papanikolopoulos · D. N. Rigopoulos
Dept of Internal Medicine, 401 GA Hospital,
Athens, Greece

D. Tsounis
Dept of Gastroenterology, 251 AF Hospital,
Athens, Greece

A. Karameris · C. Kravvaritis
Dept of Pathology, 417 VA Hospital,
Athens, Greece

A. Soultati · S. Dourakis
Dept of Gastroenterology, Hippokraton H, Medical School,
University of Athens,
Athens, Greece

T. Sergentanis · E. Patsouris
Dept of Pathology, Medical School,
National & Kapodistrian University of Athens,
Athens, Greece

A. Koliopoulou
Dept of Thoracic Surgery, Evangelismos Hospital,
Athens, Greece

E. Tsiambas (✉)
Dept of Molecular Cytopathology, 417 NIMTS Hospital,
Patriarchou Grigoriou E 17,
Ag Paraskevi Attiki 15341, Greece
e-mail: tsiambasecyto@yahoo.gr

to the gene copies ($p=0.020$). EGFR gene numerical alterations –although rare– and also chromosome 7 aneuploidy maybe affect prognosis in HCC patients. To our knowledge this is the first chromogenic *in situ* hybridization analysis based on tissue microarrays in hepatocellular carcinoma.

Keywords Chromogenic *in situ* hybridization · Epidermal growth factor receptor · Hepatocellular carcinoma · Tissue microarrays

Introduction

Hepatocellular carcinoma (HCC) is a highly aggressive and chemo resistant type of cancer. It is estimated to be the fifth most common solid malignancy worldwide and also the third cause of mortality among deaths referred to cancer [1, 2].

Epidermal growth factor receptor (EGFR) gene is located on chromosome 7 (7p12) and its product is a 170 kDa protein, comprising of three major functional domains: an extracellular ligand-binding domain, a hydrophobic transmembrane bridge and a cytoplasmic sequence of tyrosine-kinase chains [3]. Ligands, such as EGF or TGF- α , bind to the extracellular domain of the receptor and trigger a cataract of reactions, including dimerization and phosphorylation of the intracellular domain and finally signal transduction to nucleus is mediated by the involvement of RAS/RAF/MAPK proteins predominantly and also via an alternative pathway (PI3/AKT/mTOR) [4]. In aggressive tumors, such as glioblastomas, some studies have shown that EGFR gene amplification is correlated to shorter survival time and resistance to radiotherapy [5]. Almost recently, novel targeted therapeutic strategies including anti-EGFR agents, such as monoclonal antibodies (cetuximab) and small molecules (gefitinib, erlotinib) have been approved for the treatment in some types of EGFR-dependent (EGFR over expressed) cancers, such as colon or lung and pancreatic [6–8].

Although EGFR protein overexpression is observed in significant proportions of different histological origin cancers, the crucial process for a successive targeted therapeutic approach (increased response rates and extended survival) remains the identification of specific gene deregulation mechanisms. Some studies have already shown the association between specific mutations of EGFR tyrosine-kinase intracellular chains and the response to small molecules acting as inhibitors in this domain, such as gefitinib or erlotinib [9, 10]. In contrast, there are controversial results regarding to the efficacy of monoclonal antibodies therapy in those patients [11, 12]. Concerning HCC, EGFR overexpression is a frequent event and appears to be correlated to an aggressive phenotype

[13, 14]. Moreover there is a strong evidence that EGFR deregulation is associated to hepatocarcinogenesis because of it's overexpression in pre-malignant liver nodules [15].

Using tissue microarrays (TMAs) and computerized image analysis (CIA) we evaluated EGFR protein expression by Immunohistochemistry (IHC) and also we analyzed EGFR gene and also chromosome 7 status by Chromogenic *in situ* Hybridization (CISH) in order to identify potential prognostic significance of those markers in HCCs. To our knowledge this is one of the earliest studies based on these combined techniques in HCCs.

Materials and Methods

Tissue Samples

We used for the purposes of our study thirty-five ($n=35$) formalin fixed and paraffin embedded archival tissue samples of histologically confirmed HCCs. The Department of Pathology (417 VA Hospital-NIMTS, Athens, Greece) the local ethical committee gave permission to use those tissues for research purposes. Oral informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the “World Medical Association Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects” adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo 2004. Fifteen ($n=15$) cancerous tissues were obtained by the performance of percutaneous CT guided core biopsies, whereas the rest ($n=20$) were referred to local or extended liver surgical resections (lobectomies). All corresponding Hematoxylin and Eosin (H&E)–stained slides were reviewed by two pathologists for the confirmation of diagnosis and classification according to World Health Organization (WHO 2000) HCC grading criteria. The majority of them were characterized histologically by trabecular, pseudoglandular and acinar or compact architectural patterns. The tissue samples were referred to 26 male (mean age: 57) and 9 female (mean age: 62) patients followed-up in a period of 6 to 18 months after diagnosis. Clinicopathological data are demonstrated in Table 1.

TMA Construction

Areas of interest were identified in H&E stained slides by a conventional microscope (Olympus BX-50, Melville, NY, USA). Selection of those areas was performed on the basis of tumor sufficiency avoiding sites of necrosis or bleeding. Using ATA-100 apparatus (Chemicon International, Temecula, CA, USA), all of the source blocks were cored two times (in order to secure the presence of each case in the final block) and 1.5 mm diameter tissue cylindrical cores

Table 1 Clinicopathological features of the examined HCCs

<i>n</i> =35	
Gender	
Female	(<i>n</i> =9)
Male	(<i>n</i> =26)
Grade	
I	(<i>n</i> =11)
II	(<i>n</i> =16)
III	(<i>n</i> =8)
Stage	
I	(<i>n</i> =5)
II	(<i>n</i> =18)
III	(<i>n</i> =10)
IV	(<i>n</i> =2)
History	
HBV	(<i>n</i> =21)
HCV	(<i>n</i> =4)
Non-HV	(<i>n</i> =10)
Survival	
Dead	(<i>n</i> =20)
Alive	(<i>n</i> =15)
Survival rates (in months)	
0–6	(<i>n</i> =8)
>6–12	(<i>n</i> =21)
>12	(<i>n</i> =6)

were transferred from each conventional donor block to the recipient block. After 3 mm microtome sectioning and H&E staining, we observed microscopically that all examined cases were represented by at least one (12 cases) or two tissue spot (23 cases)-confirmation of the adequacy of specimens.

Antibodies and Probes

Ready-to-use EGFR monoclonal mouse antibody (clone 31G7-Zymed/InVitrogen, San Francisco, USA) recognizing predominantly the extracellular domain of EGFR protein and not reacting with other erbB receptors was applied for the identification of protein expression. EGFR gene status was determined using the ready to use SPOT LIGHT EGFR DNA Probe (Zymed/InVitrogen, San Francisco, USA). This digoxigenin-labeled probe is located on 7p12 and covers the entire EGFR gene area. Similarly, chromosome 7 status was determined by the ready to use biotin-labeled chromosome 7 centromeric probe (Zymed/InVitrogen, San Francisco, USA) recognizing the specific repetitive centromeric DNA sequences known as α -satellite DNA.

Immunohistochemistry (IHC)

IHC for EGFR antigen was carried out on 3 μ m serial sections. Two slides were deparaffinized and rehydrated. Both of them

were enzyme digested (proteinase K) for 10 min at 37°C. The NBA kit (Zymed/InVitrogen, San Francisco, USA) was used for the following detection steps. Blocking solution was applied to the slides for 10 min, followed by incubation for 1 h using the EGFR monoclonal antibody (dilution 1:10) at room temperature. Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrochloride-DAB (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. For negative control slides, the primary antibody was omitted. IHC protocol was performed by the use of an automated staining system (I 6000-Biogenex, San Ramon, CA, USA). Membranous predominantly and sub-membranous cytoplasmic staining was considered acceptable for EGFR expression according to the manufacturer's data sheet (Fig. 1 a). Colon cancer tissue sections over expressing EGFR and normal appearing colon epithelia were used as a positive and negative control, respectively. At first, EGFR protein expression levels were evaluated semi-quantitatively by using Zymed's Evaluation Guidelines. According to the scoring guidelines, the examined cases were classified as follows: Score 0: no staining or membrane staining in <10% of tumor cells; Score 1+: faint membrane staining in >10% of tumor cells; Score 2+: weak or moderate complete membrane staining in >10% of tumor cells and Score 3+: strong, complete membrane staining in >10% of tumor cells. Scores of 0 and 1+ were considered as negative for EGFR expression while Scores 2+ and 3+ as positive (overexpression).

Chromogenic *in situ* Hybridization (CISH)

CISH SPOT-Light Chromogenic ISH Detection Kit (Zymed/InVitrogen, USA) was applied. CISH for chromosome 7 status and EGFR gene analysis was performed on 5 μ m thick paraffin serial sections of the TMA block described above. Two slides were incubated at 37 °C overnight followed by 2 h incubation at 60 °C and then deparaffinized in xylene two times, 5 min each and in ethanol three times, 3 min each. The slides were rinsed in deionised water and then placed in a coplin jar containing CISH FFPE Pre-treatment Buffer (CISH Tissue Pre-treatment Kit, Zymed). For heat pre-treatment, the coplin jar was capped, loosely screwed, placed in a pressure cooker and timed for 10 min after the pressure built up. The slides, then, were immediately washed in deionised water followed by enzyme digestion, which was performed by covering the sections with pepsin (CISH Tissue Pre-treatment Kit, Zymed) for 5 min at 37 °C. The slides were washed with deionised water, dehydrated with graded ethanol and air-dried. Ready to use dig-labeled EGFR gene and biotin-labeled chromosome 7 centromere probe were applied to each section, respectively. Twenty microliter of probe was applied to each of the TMA sections. The

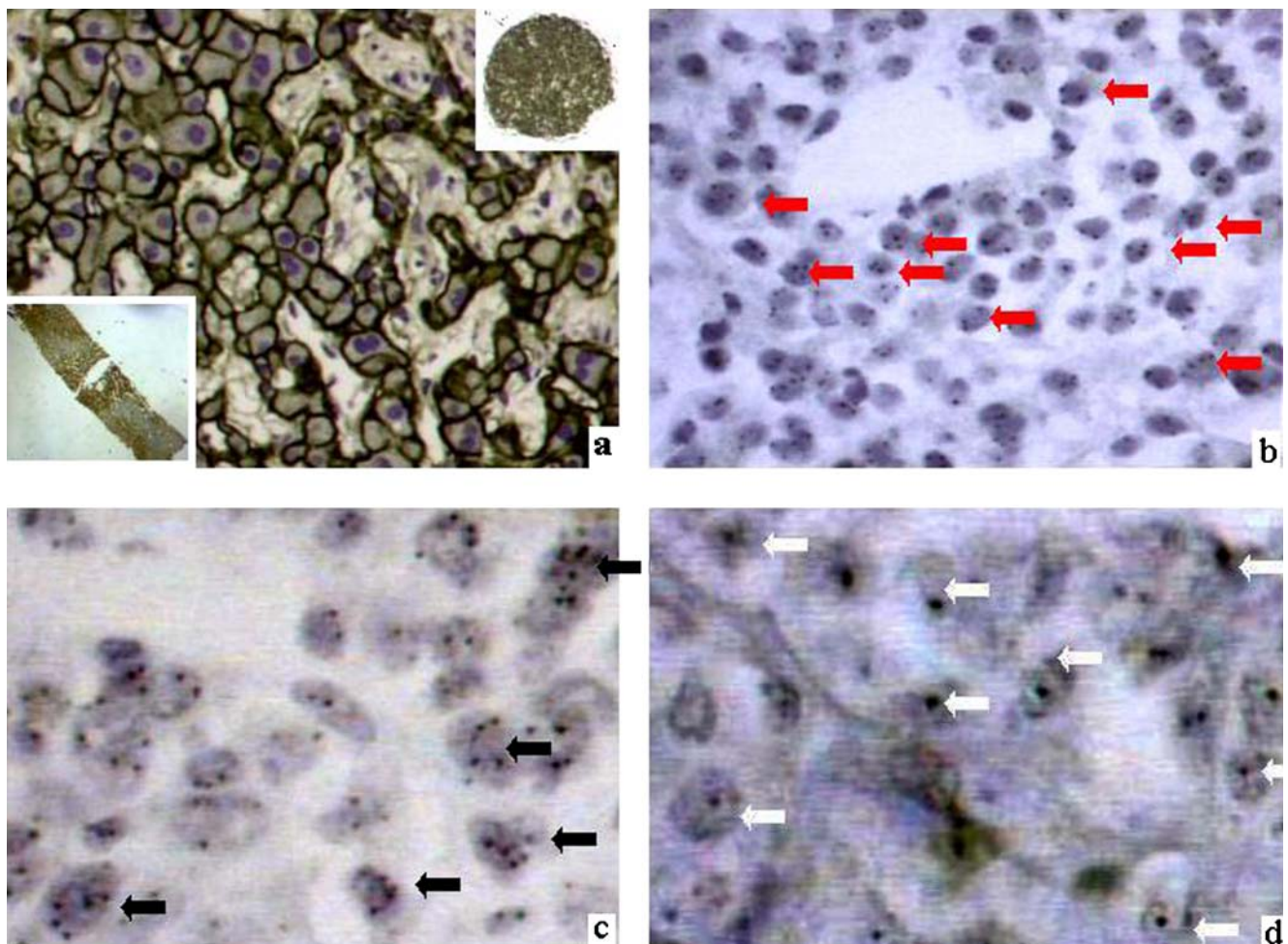


Fig. 1 **a** EGFR protein overexpression in a tissue microarray core and also in an FNAB biopsy of HCC. Note EGFR membranous “ring-like”, dense staining pattern (original magnification 40×), **b** Chromosome 7 numerical alterations (aneuploidy) detected by CISH method. Note 3–5 centromeric copies per nucleus (red arrows, original

magnification 40×), **c** EGFR gene low amplification. Note 5 to 7 scattered gene copies per nucleus (black arrows, original magnification 40×), **d** EGFR allelic absence. Note 1 gene copy per nucleus (white arrows, original magnification 40×)

tissue sections containing the added probe were denatured by placing the slides in a polymerase chain reaction (PCR) machine equipped with a slide block at 94 °C for 5 min. The slides were then placed in a moist slide box and incubated at 37 °C for overnight hybridization. The sections were stringently washed in 0.5x standard saline citrate at 75 °C for 5 min. The CISH Polymer and the Horseradish (HRP) Detection Kit (Zymed/InVitrogen, San Francisco, USA)–containing similar steps to IHC–were used. Shortly afterwards TMA sections were placed in 3% H₂O₂ and diluted with methanol for 10 min to block endogenous peroxidase. To block unspecific staining, Cas Block™ (Zymed/InVitrogen, San Francisco, USA) was applied and incubated for 10 min. Following incubation with mouse anti-dig for 30 min and then polymerised HRP conjugated anti-mouse for 30 min, the EGFR probe was visualized by DAB

development (CISH Polymer Detection Kit, Zymed). The biotin labeled Chr 7 centromere probe was detected by incubation with HRP conjugated streptavidin for 30 min, followed by DAB development (CISH Centromere Detection Kit, Zymed) for 30 min. TMA sections were lightly counterstained with hematoxylin and dehydrated in graded ethanol. At the end of the process, CISH centromere signals or gene copies were easily visualized as dark brown/blue scattered or in small clusters dots, using a conventional, bright-field microscope (Fig. 1 b). Interpretation of EGFR gene and chromosome 7 centromere signal results was based on Zymed’s Evaluation Chart for CISH. According to this guide, two gene copies per nucleus demonstrate normal EGFR gene pattern, whereas 6–10 or small clusters characterize a low-level gene amplification. In this case, chromosome 7 status must be evaluated to exclude aneu-

ploidy (3–5 centromeric signals per nucleus; diploid pattern demonstrates normal chromosome status). High gene amplification level is characterized by the presence of more than 10 gene copies or large clusters of them per nucleus in more than 50% of the examined cells, whereas the presence of a smaller number of EGFR copies than chromosome 7 centromeric signals is considered to be an evidence of gene deletion or “silence” due to mechanisms, such as point mutation or loss of heterozygosity (LOH).

Computerized Image Analysis (CIA)

In order to evaluate the IHC results—specifically 2+ and 3+ semi-quantitatively determined cases—in an accurate and faster way, we perform CIA by using a semi-automated system with the following hardware features: Intel Pentium IV, Sony camera (800×600), Microscope Olympus BX-50 and the following software: Windows XP (Microsoft USA) / Image Pro Plus, version 3.0-(Media Cybernetics 1997). Measurements of EGFR staining intensity values were performed in five optical fields per case and at a magnification of 400× (Fig. 2). All measurements were performed inside an active window of 16,848 μm^2 . All numerical data were filed on Microsoft Excel sheets. Interpretation of staining intensity values (range 0–255) is demonstrated in Table 2.

Statistical Analysis

Associations between variables including protein expression levels, gene and chromosome 7 alterations and clinicopathological parameters were performed by the application of STATA 8.0 statistical software (Stata Corporation, College Station, TX, USA). Two tailed *p* values <0,05 were considered to be statistically significant. EGF-R expression was treated as an ordinal variable (1: low, 2: moderate, 3: high), whereas *egf-r* gene and chr7 chromosome status were treated as binary variables. For the evaluation of the effect mediated by putative predictors (EGF-R expression, chr7 status, *egf-r* gene status, gender, grade, and hepatitis status) upon patients' survival, univariable Cox regression modeling was performed and also Kaplan-meier's curves were developed for survival analysis (Fig. 3). Hazard ratios (HR), together with the 95% confidence intervals (CI) are reported. The current sample did not permit the multivariable evaluation of results. Due to the relatively small sample size, *p*-values of borderline significance are also reported, since they may become statistically significant in the context of larger samples. Also kappa analysis was performed regarding conventional and digital image analysis methods of EGFR protein expression evaluation. Total IHC and CISH results and also *p* values are described in Table 2.

Results

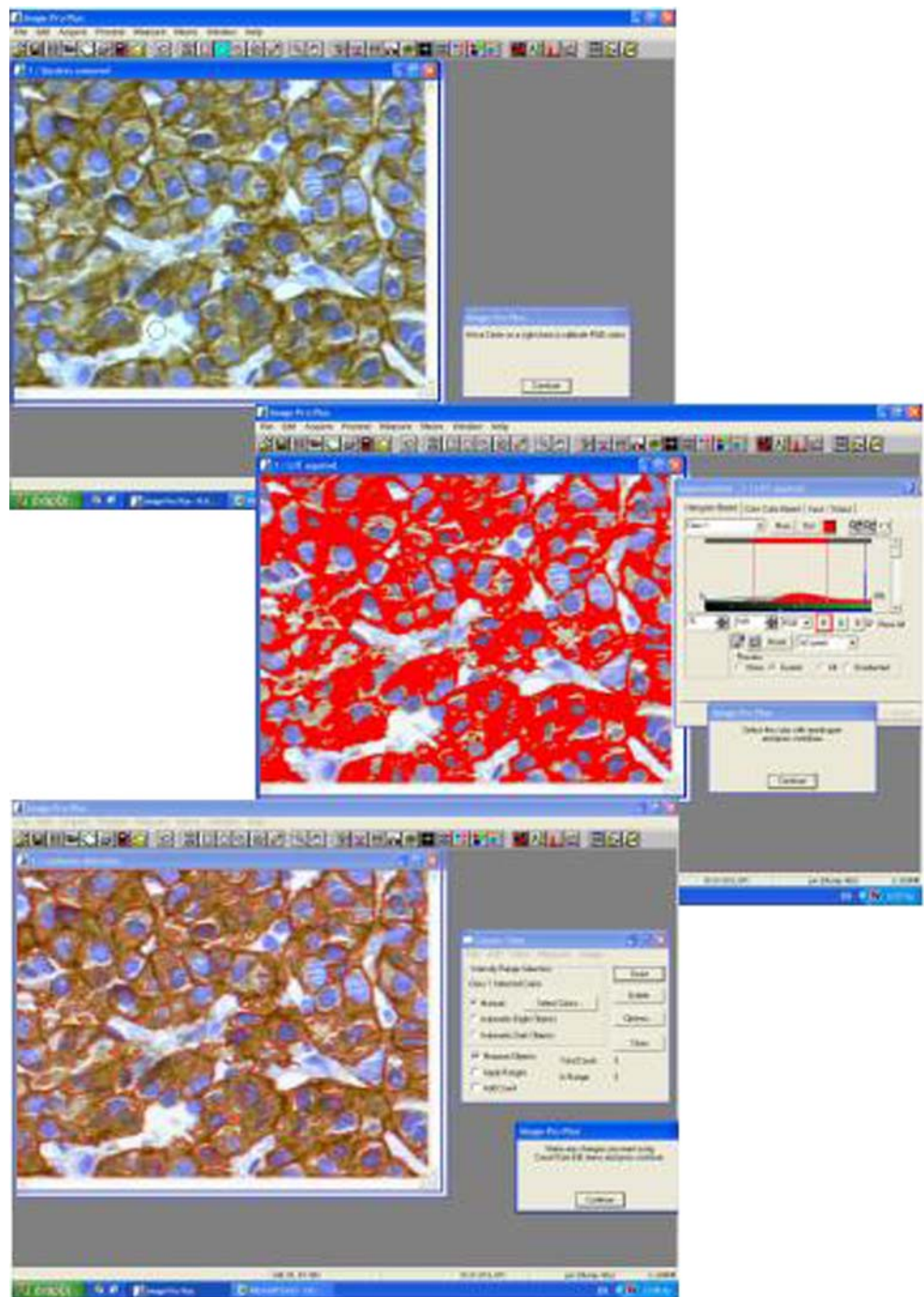
IHC results were successfully obtained from all HCC cases. EGFR overexpression was observed in 26/35 (74.2%) cases, whereas the rest of them (*n*=9) were characterized by low or negative protein expression. According to the conventional evaluation criteria, 19 cases were evaluated as 3+ and 7 cases as 2+. Computerized image analysis for EGFR protein staining intensity levels showed that 14 cases demonstrated high values, whereas 12 cases moderate values. EGFR protein expression was statistically associated with grade (*p*=0.013), but not with stage (*p*=0.233) or gender (*p*=0.123) of the examined tumors.

CISH results were also successfully obtained from all examined cases. In the majority of them (*n*=21), EGFR gene was observed to be normal (2 gene copies per nucleus), whereas the rest of the analyzed cases (*n*=14) demonstrated numerical alterations. In ten (*n*=10) of them 3 to 5 gene copies per nucleus were identified, in three of them (*n*=3) only one copy per nucleus and in one case, 6 to 7 copies per nucleus of EGFR gene were detected. In order to determine the exact genetic event regarding EGFR gene numerical alterations cases, we analyzed the chromosome 7 status. Chromosome 7 instability (aneuploidy / polysomy, observed as 3–5 dots per nucleus) was detected in 12/35 (34.2%) cases, whereas 23/35 (65.8%) cases demonstrated a normal, diploid pattern (2 centromeric copies per nucleus). We observed that in two cases (*n*=2) of chromosome 7 polysomy and in one of normal pattern, EGFR gene copies were lesser than the corresponding centromeric signals. Additionally, in one case characterized by chromosome 7 normal, diploid pattern, multiple EGFR copies were detected. Significant statistical correlation was observed by associating overall EGFR gene copy number to the grade of the tumors (*p*=0.019) but not to the stage or gender of them (*p*=0.132, and *p*=0.947, respectively). Interestingly, by correlating overall EGFR expression to gene status, we observed a strong statistical significance (*p*=0.020), whereas a borderline significance was assessed associating protein expression and chromosome 7 status (*p*=0.061). Furthermore, we did not observe a significance, correlating gene and chromosome status (*p*=0.537).

Concerning potential correlation of the history of the examined patients with HCC to EGFR alterations, protein expression, especially high levels, was significantly associated to viral infection (*p*=0.036). The majority of the highly overexpressed cases were found to be HBV infected, whereas EGFR gene and also chromosome 7 numerical alterations did not demonstrate a significant correlation to HBV infection (*p*=0.097 and *p*=0.815, respectively).

Survival analysis showed that *Egf-r* gene aberrations (deletion / amplification) were associated with shorter

Fig. 2 Computerized image analysis of EGFR protein expression levels. Reddish areas represent the membranous/sub membranous levels of staining intensity (original magnification 40 \times)



patients' survival (HR=3.39, 95%CI: 1.08–10.65, $p=0.037$) (Fig. 3 a). Chr7 aneuploidy was also associated with shorter patients' survival (HR=6.06, 95%CI: 2.21–16.63, $p<0.001$) (Fig. 3 b). Higher tumour grade was also associated with decreased survival (HR=3.42, 95%CI: 1.68–6.96, $p=0.001$). On the other hand, patient's survival was not associated with patients' gender, EGFR expression, and tumour stage or hepatitis status.

Discussion

Extensive cytogenetic analyses have shown that hepatocarcinogenesis represents a multi-step process depended on the progressive accumulation of genetic alterations in normal hepatocytes [16]. Although dysplastic cirrhotic nodule is the histological substrate for the malignant transformation in the majority of HCCs, it is considered that different

Table 2 IHC and CISH results and correlations with clinicopathological parameters

	EGFR protein*			p	EGFR gene		p	Chr7		p
	Low (0/1+) (n=9)	Moderate (n=12)	high (n=14)		normal (n=31)	aberration (n=4)		normal (n=23)	aneuploidy (n=12)	
EGFR gene	0.020^{MWW}									
normal (n=31)	29.0% (9/31)	38.7% (12/31)	32.3% (10/31)							
aberration (n=4)	0% (0/4)	0% (0/4)	100% (4/4)							
Chromosome 7	0.061^{MWW}									
normal (n=23)	34.8% (8/23)	34.8% (8/23)	30.4% (7/23)		91.3% (21/23)	8.7% (2/23)				
aneuploidy (n=12)	8.3% (1/12)	33.3% (4/12)	58.3% (7/12)		83.3% (10/12)	16.7% (2/12)				
Grade	0.013^S									
1 (n=11)	45.5% (5/11)	36.4% (4/11)	18.2% (2/11)		100% (11/11)	0% (0/11)	0.019^{MWW}	100% (11/11)	0% (0/11)	0.0001^{MWW}
2 (n=16)	18.7% (3/16)	43.8% (7/16)	37.5% (6/16)		93.8% (15/16)	6.2% (1/16)		68.8% (11/16)	31.2% (5/16)	
3 (n=8)	12.5% (1/8)	12.5% (1/8)	75.0% (6/8)		62.5% (5/8)	37.5% (3/8)		12.5% (1/8)	87.5% (7/8)	
Hepatitis status	0.036^{MWW}									
negative (n=10)	40.0% (4/10)	50.0% (5/10)	10.0% (1/10)		100% (10/10)	0% (0/10)		60.0% (6/10)	40.0% (4/10)	
HBV/HCV (n=25)	20.0% (5/25)	28.0% (7/25)	52.0% (13/25)		84.0% (21/25)	16.0% (4/25)		68.0% (17/25)	32.0% (8/25)	

NS statistically not significant, S p-value derived from Spearman’s rank correlation coefficient, M p-value derived from Mann-Whitney Wilcoxon test for independent samples (EGF-R expression and grade treated as ordinal variables)

EGFR protein*: Protein expression estimated as Low, Moderate and High according to image analysis (Moderate: 114–129, High: 77–98 in a range of 0–255 values of staining intensity). Low expression is equally to conventional 0/1+ evaluation

genes are altered by HBV and HCV infection, respectively [17]. HBV-related carcinogenic process is characterized predominantly by chromosome instability including deleted chromosome arms, such as 17p, 13q, 16p, 16q, 4q and also gains of 1q [18]. Inactivation of important suppressor genes, such as Tp53 and retinoblastoma due to loss of heterozygosity (LOH) and also axis inhibition protein-1 due to point mutations are associated to this deregulation mechanism [19]. Non-HBV-dependent HCCs demonstrate different molecular alterations correlated to chromosome stability and combined to specific gene instabilities such as b-catenin/wnt pathway and k ras oncogene activation due to point mutations [20, 21]. In contrast, there are controversial and limited data regarding EGFR specific gene deregulation mechanism in HCCs and the exact correlation with the viral infection [22, 23].

In the current pilot study (n=35 patients), we analyzed EGFR at the protein and also at the DNA level based on tissue microarrays and also on core biopsy specimens. The

majority of the examined cancerous subpopulations demonstrated overexpression of the marker, whereas only a limited subset of them was characterized by specific gene numerical alterations (gain or absence/loss) and also chromosome 7 instability (aneuploidy) but affecting the survival status of the patients (poor prognosis). Furthermore, statistical significance was established correlating protein expression and gene/chromosome numerical aberrations with the grade of the tumors, whereas only EGFR expression was associated to the history of the patients. Additionally, a significant correlation was observed between EGFR protein expression levels and gene status. Interestingly, the stage of the tumors was not associated to the overall survival of the examined patients. Although this result is not in correlation with the majority of the data in the literature it could be explained by the limited number of the examined cases that demonstrated the most advanced stage (only 2 out of 35 cases). This event sometimes affects the statistical analysis. According to our CISH results,

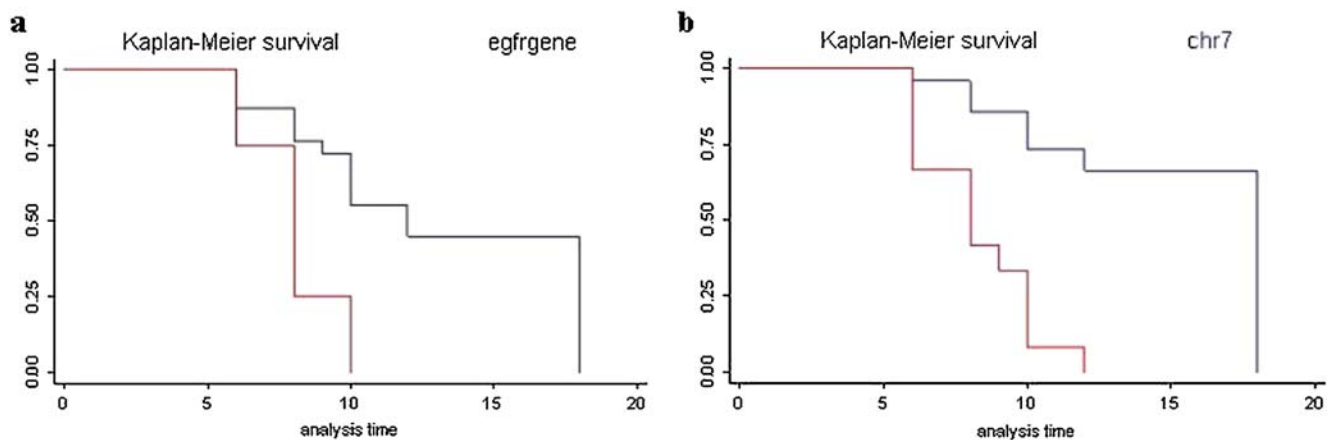


Fig. 3 Kaplan-Meier curves for the effect of gene **a** and chromosome 7 **b** aberrations. Blue line: normal status. Red line: aberration carriers (gene gain/loss, chromosome 7 aneuploidy)

HCCs are characterized by different molecular profiles of EGFR gene instability including potential gene deletion or alternatively gene “silence” due to loss of one allele or to point mutations, whereas low gene amplification is a very rare event. Some recently published studies based on Polymerase Chain Reaction analysis (PCR assays) have failed to detect specific mutations affecting the catalytic domain of the tyrosine-kinase chains [24, 25]. In contrast to this, a novel somatic mutation in HER2/neu, another member of the EGFR type I protein super family, was identified in HCCs (H878Y: CAT to TAT; c.2632C > T mutation in exon 21) [26]. The lack of EGFR gene point mutations in HCCs provides a skepticism regarding the response of patients with HCC to anti-EGFR tyrosine-kinase inhibitors, such as gefitinib or erlotinib [27, 28]. Furthermore, targeted therapeutic regimens based on a combination of cetuximab and erlotinib in experimental studies decreases the proliferation and also increases apoptosis by the induction of its positive regulators [29].

EGFR gene amplification combined with protein overexpression is a relatively frequent event in colon adenocarcinoma, in non-small cell lung carcinoma, but not in HCCs [30]. Almost recently a study showed that fibrolamellar hepatocellular carcinoma—a non-cirrhotic depended type of liver neoplasm affecting predominantly young patients—not only overexpresses significantly the marker but also is characterized by accumulated EGFR copies in the majority of the examined cases [31]. Using fluorescence in situ hybridization (FISH) the study group analyzed also the chromosome 7 status and observed that the extra gene copies were correlated to gains of the chromosome and not to a real amplification mechanism. In our study, chromosome 7 analysis detected only one case characterized by an EGFR/CEP 7 ratio of 1.9 and reflecting a low amplification profile. In agreement to our chromosome 7 analysis, related studies have showed that chromosome 7 instability also is

correlated to tumor dedifferentiation and poor prognosis in HCCs [32].

The significance of this EGFR gene deregulation mechanism (gene amplification) regarding the response to anti-EGFR targeted therapeutic strategies is under investigation. It seems that EGFR protein overexpression alone is not the eligible criterion in selecting patients for the application of monoclonal antibody inhibition due to binding on the receptor extracellular domain [10, 33]. Furthermore, EGFR gene amplification in patients with colon adenocarcinoma was associated to survival benefits (extended life span). Additionally, alternative mechanisms of EGFR activation have been identified. In one of them, the recently cloned EGFR-related protein that regulates the expression levels of EGFR frequently decreases in HCCs affecting indirectly the levels of EGFR immunostaining [34]. Concerning the correlation between viral infection and EGFR expression we observed that tissue samples of the HBV infected patients expressed the highest levels of the protein as was determined by the image analysis procedure (staining intensity levels). Related studies observed that HBV integrated X region provides malignant transformation in those carriers and increases the EGFR m-RNA levels [35].

In the current technical paper, we performed a computerized image analysis assay in order to evaluate the EGFR protein expression results by a rapid, accurate and quantitative method. Comparing the results of the conventional eye-microscopy evaluation and the corresponding of digital analysis we observed some differences especially in the borderline (2+/3+) overexpression cases. Digital analysis detected specific staining intensity levels in contrast to human eye, which is characterized by limitations in discriminating those levels. This procedure provides a fast and accurate evaluation and its applications are increased in the medical literature, especially in the field of diagnostic

pathology and focused on EGFR or HER2/neu evaluation as a basis for targeted therapy [36–38]. Concerning EGFR protein image analysis in HCCs, our recently presented study confirmed the benefits of the method [39].

In conclusion, a small subset of HCCs is characterized by numerical aberrations regarding EGFR gene and also chromosome 7 that affect potentially the prognosis of the patients. Gene deregulation mechanism and not only EGFR protein overexpression maybe is the eligible approach for a rational anti-EGFR targeted therapeutic strategy in HCCs including potentially novel agents such as lapatinib or sorafenib, dual inhibitors of both EGFR- and HER2-associated tyrosine kinases or Raf kinase and VEGF / PDGF receptors, respectively [40, 41].

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Conflict of interest statement The authors declare no conflict of interest.

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