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ORIGINAL PAPER

Significance of estrogen receptor 1 (ESR-1) gene imbalances in colon and hepatocellular carcinomas based on tissue microarrays analysis

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Abstract Estrogen receptor alpha–encoded by ESR1 gene–overexpression correlates with prognosis and response to specific chemotherapy in breast adenocarcinoma cases. Mechanisms of ESR-1 deregulation in carcinomas remain under investigation. To analyze ESR1 in carcinomas of different histogenesis. Using tissue microarray technology, 172 primary carcinomas including breast ductal adenocarcinomas ($n = 60$), hepatocellular carcinomas ($n = 52$), and colon adenocarcinomas ($n = 60$) were cored and re-embedded in three paraffin blocks. Initial diagnosis was based on liquid based cytology (LiquiPrep/ThinPrep). Immunohistochemistry and fluorescence in situ hybridization were performed. Quantitative evaluation of ER-a protein levels was assessed by applying digital image analysis. ER-a overexpression was observed in 41/60 (68.3%), 23/52 (44.2%) and 4/60 (6.6%) cases, respectively. ESR1 gene multiple copies were confirmed in 13/60 (21.6%) breast adenocarcinomas, but high amplification only in 8/13 (62.8%). Allelic absence was identified in 3/52 (5.7%) hepatocellular carcinomas, whereas colon adenocarcinomas demonstrated gene gains in 5/60 (8.3%) cases referred to chr 6 aneuploidy and not to amplification. ER-a overall expression was associated strongly to ESR1 gene copies only in breast carcinoma ($P = 0.036$). ESR-1 gene overexpression happens frequently in breast cancer, but only a subset of them are high amplified cases correlated to increased response rates in hormonal therapy (tamoxifen). Absence of this mechanism in hepatocellular and colon carcinomas maybe is a negative factor for applying this therapy. This is a pattern of histo-genetic depended targeted therapeutic strategy.

Keywords Estrogen receptor · Gene · Amplification · Microarrays · Liquid based cytology

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Introduction

Colon adenocarcinoma, breast carcinoma and also hepatocellulatr carcinoma are leading causes of cancer death worldwide [1, 2]. Extensive cytogenetic analyses have confirmed the crucial role of specific genes that are deregulated in the carcinogenetic and metastatic process regarding those malignancies [3, 4]. Concerning breast adenocarcinoma, HER2/neu, Topoisomerase IIa and estrogen/progesterone receptors are frequently altered and over expressed affecting also the biological behavior of the neoplasm [5, 6].

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Estrogen receptors represent a family of proteins activated by the hormone 17β-estradiol. There are two types of receptors: the ER intracellular/nuclear receptors which demonstrate two isoforms (ESR-1 and ESR-2) and the estrogen G-protein coupled receptor [7]. ESR-1 gene located on chromosome 6 (6q25.1) encodes estrogen receptor a (ER-a) protein that acts as a strong DNA binding transcription factor regulating gene expression [8]. Besides the significant role of estrogens/estrogen receptors complexes in normal physiology of various organs other than those of the reproductive system, they are also involved in key functions, such as generation of immune responses, cell proliferation and apoptosis, even in bone metabolism [9].

ER-a over expression due to ESR-1 gene amplification seem to affect the response rates in breast cancer patients that receive targeted hormone-based monotherapy or adjuvant therapy (tamoxifen) [10].Furthermore, there is a strong evidence that tamoxifen-resistant caner cells have the ability to switch between ERBB2 or ESR1 depended pathways [11]. In contrast to breast cancer therapeutic experience, there are very limited data regarding the ESR-1 deregulation and the potential usefulness of tamoxifen application in patients with gastrointestinal malignancies, such as hepatocellular carcinoma and colon adenocarcinoma [12].

In the current basic research study, we analyzed estrogen receptor at the DNA and protein expression level in order to identify potential association between gene numerical imbalances and different expression profiles in those malignancies.

Materials and methods

Tissue samples

We used 172 ($n = 172$) formalin fixed and paraffin embedded tissue samples of histologically confirmed carcinomas of different histological origin (breast ductal adenocarcinomas $n = 60$, hepatocellular carcinomas $n = 52$, and colon adenocarcinomas $n = 60$) derived from histological base of Dept of Pathology, 417 VA Hospital (years 2002–2009). All research protocols were performed according to ethical guidelines of the "World Medical Association Declaration of Helsinki–Ethical Principles for Medical Research Involving Human Subjects". All corresponding Hematoxylin and Eosin (H&E)-stained slides were reviewed by two pathologists for confirmation of diagnosis and classification according to World Health Organization (WHO 2000/2002) grading and staging criteria for the gastrointestinal and breast carcinomas, respectively [13, 14]. Clinicopathological data are demonstrated in Table 1.

Tissue microarrays construction

Areas of interest were identified in H&E stained slides by a conventional microscope (Olympus BX-50, Melville, NY, USA). Using ATA-100 apparatus (Chemicon International, Temecula, CA, USA), all of the source blocks were cored and 1.5 mm diameter tissue cylindrical cores were transferred from each conventional donor block to the recipient blocks ($n = 3$) (Fig. 1).

Cytological specimens' management

The initial diagnosis of breast cancer cases was provided by applying fine needle aspiration (FNA). Diagnostic evaluation was assessed via liquid based cytology (ThinPrep method, Cytyc, CA, USA and also LiquiPrep, Melbourne, FL, USA). All the cytological samples were stained by Papanicolaou staining method.

Immunohistochemistry (IHC)

Ready-to-use anti-ER-a mouse monoclonal antibody (NCL-L-ER-6F11, Novocastra, UK-dilution 1:100) was applied for detecting protein expression. IHC protocol was performed by the use of an automated staining system (Bond–Biogenex, San Ramon, CA, USA). Nuclear predominantly and also perinuclear-cytoplasmic staining was considered acceptable for the marker (Fig. 1). Breast cancer tissue sections expressing the protein and normal appearing breast epithelia were used as control groups. Criteria for ER-a overexpression are described in Table 1.

Computerized image analysis assay (CIA)

ER-a protein expression levels were evaluated by a combination of two parameters: staining intensity levels provided by digital image analysis and the percentage of stained nuclei. We performed CIA using a semi-automated system (Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Nuclear Labelling Index (NLI) was estimated by conventional microscopy (Fig. 2).

Fluorescence in situ hybridization (FISH)

ESR-1 gene and chromosome six analysis was performed by applying an ESR-1/CEN six dual color probe kit (Zytolight^{TM}SPEC, Zytovision, Ge) recognizing the specific gene and repetitive centromeric DNA sequences known as α-satellite DNA (Fig. 3). Evaluation of the visualized ESR1/CEN 6 signals on the slides was performed immediately after their construction by a fluorescence microscope (Olympus, Menville, NY, USA). ESR1/CEN 6 ratios were calculated based on the modified instructions for FISH HER2/neu

Table 1 Combined IHC & FISH results and *P* values

Histology $n = 172$	ER-a protein			ESR-1 gene		
	Low expression	Overexpression	*P***	Non gene aberration*	Aberration (Amplification allelic absence)	*P***
Breast adenocarcinoma						
($n = 60$)	$n = 19$	$n = 41$		$n = 47$	$n = 13$	
Grade			0.106			0.248
1 ($n = 25$)	10/25	15/25		21/25	4/25	
2 ($n = 22$)	8/22	14/22		19/22	3/22	
3 ($n = 13$)	1/13	12/13		7/13	6/13	
Stage			0.357			0.424
1 ($n = 10$)	5/10	5/10		9/10	1/10	
2 ($n = 34$)	8/34	26/34		27/34	7/34	
3 ($n = 12$)	5/12	7/12		9/12	3/12	
4 ($n = 4$)	1/4	3/4		2/4	2/4	
Hepatocellular carcinoma						
($n = 52$)	$n = 29$	$n = 23$		$n = 49$	$n = 3$	
Grade			0.001			0.238
1 ($n = 16$)	15/16	1/16		15/16	1/16	
2 ($n = 21$)	11/21	10/21		21/21	0/21	
3 ($n = 15$)	3/15	12/15		13/15	2/15	
Stage			0.016			0.698
1 ($n = 12$)	2/12	10/12		12/12	0/12	
2 ($n = 25$)	17/25	8/25		23/25	2/25	
3 ($n = 11$)	8/11	3/11		10/11	1/11	
4 ($n = 4$)	2/4	2/4		4/4	0/4	
Colon adenocarcinoma						
($n = 60$)	$n = 56$	$n = 4$		$n = 60$	$n = 0$	
Grade			0.183			0.129
1 ($n = 36$)	35/36	1/36		36/36	0/36	
2 ($n = 20$)	18/20	2/20		20/20	0/20	
3 ($n = 4$)	3/4	1/4		4/4	0/4	
Stage			NSC			NSC
1 ($n = 22$)	22/22	0/22		22/22	0/22	
2 ($n = 28$)	26/28	2/28		28/28	0/28	
3 ($n = 7$)	6/7	1/7		7/7	0/7	
4 ($n = 3$)	2/3	1/3		3/3	0/3	

* Normal (diploid) & Gains due to chromosome 6 polysomy

** S: *P*-value derived from chi square test NSC: statistically not computed

ER-a protein expression estimated as Low, Moderate and High according to image analysis staining intensity level (Low expression: >156, Moderate: 101–151 & High: 72–91-in a range of 0–255 values of staining intensity)and also to % number of stained nuclei. Overexpression refers to >50% of stained nuclei with moderate or high staining intensity values

evaluation [15]. According to them, in the cases with scattered gene signals, an ESR1/CEN 6 ratio of 2.2 or higher was considered positive for high gene amplification, a ratio between 1.8 and 2.2 positive for low gene amplification, whereas a ratio of <1.8 was considered negative. Cases with gene clusters, were considered as amplified, whereas a ratio of 1 in the cases of equal ESR1 and CEN copies over three for both of them were considered as polysomy. Finally, cases characterized by a ratio <1–0.4 were considered as allelic absences.

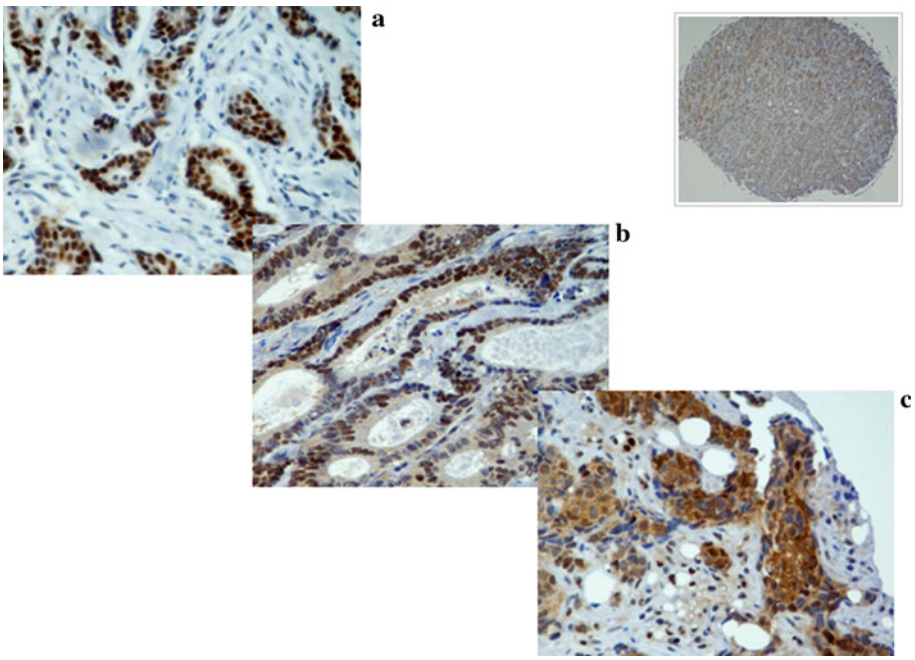

Fig. 1 Different expression patterns of ER-a in solid tumours. **a** Overexpression in breast cancer. Note strong nuclear stain (dark stained nuclei) **b**. Overexpression in a case of colon adenocarcinoma **c**. A case of hepatocellular carcinoma. Note nuclear and cytoplasmic expression colon adenocarcinoma overexpressing the marker (original magnification: 10×, inside 40×). Inside image: Overexpression in HCC

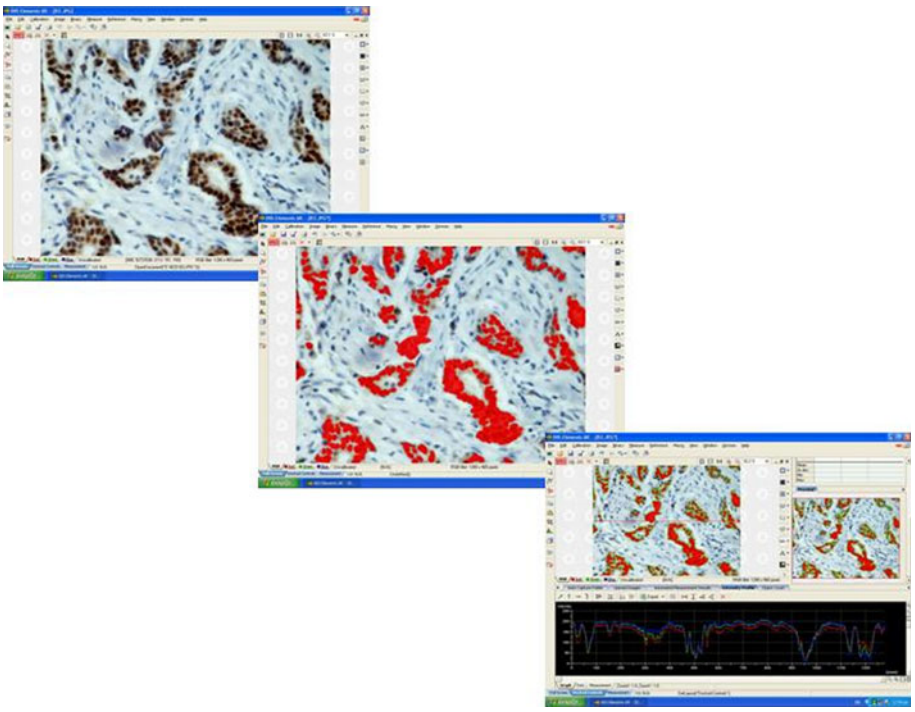

Fig. 2 Evaluation of staining intensity levels in the examined tumours by digital image analysis (NIS-elements). Dark covered areas present different ER-a protein expression values ranged between 0 and 255. Note the imbalances of staining intensity as curves in the corresponding diagrams

Statistical analysis

Associations between ER-a protein expression levels and gene/chromosome ratio alterations were performed by the application of chi-square and Fischer's tests (SPSS v 11.0 statistical software- Inc Chicago IL, USA). Total IHC and FISH results and also *P* values are described in Table 1.

Results

IHC results were successfully obtained from all analyzed cases. ER-a overexpression (high and moderate level of expression) was observed in 41/60 (68.3%) breast adenocarcinoma cases, in 23/52 (44.2%) hepatocellular carcinomas and in 4/60 (6.6%) colon adenocarcinomas, respectively.

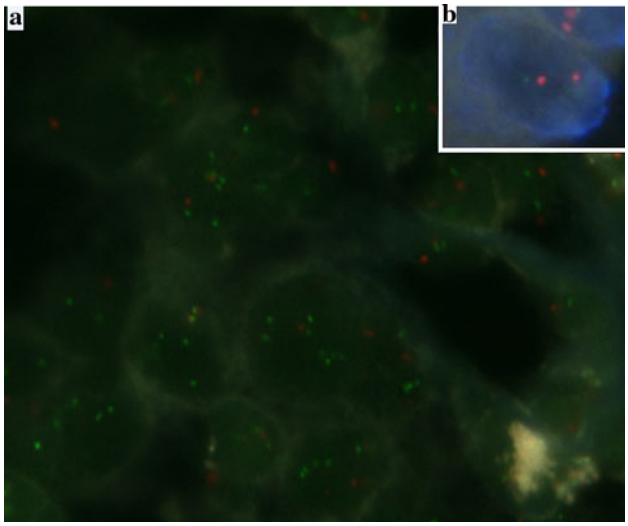

Fig. 3 Numerical imbalances of ESR-1 gene detected by FISH analysis. **a** Gene amplification in a case of breast cancer overexpressing ER-a. Note multiple signals (represent ESR-1 copies) **b**. A case of allelic absence in hepatocellular carcinoma. Note a ratio of 1:2 signals (ESR-1: Chr 6)

Furthermore, a low level ER-a expression was identified in 15/60 (25%) breast adenocarcinoma cases, in 20/52 (38.4%) hepatocellular carcinomas and in 17/60 (26.3%) colon adenocarcinomas, whereas the rest of the examined tissue cores were completely negative for the marker. ER-a overall expression was strongly correlated with grade and also stage of the examined hepatocellular carcinomas ($P = 0.001$, $P = 0.016$, respectively).

FISH results were also successfully obtained from all examined cases. In the majority of them ($n = 135$), ESR-1 gene was observed to be normal (ESR-1/CEN 6 ratio = 1, 2 gene and also equal to them chromosome centromere copies), whereas the rest of the analyzed cases ($n = 37$) demonstrated numerical alterations. Concerning breast adenocarcinoma, ESR-1 gene amplification (ESR-1/CEN 6 ratio >1.8) was detected in 13/60 (21.6%) cases, but only in eight ($n = 8$) out of them a high gene amplification profile was confirmed (ESR-1/CEN 6 ratio >2.2). Additionally, in six ($n = 6$) tissue cores chromosome 6 polysomy (ESR-1/CEN 6 ratio = 1, 3–5 gene and also equal to them chromosome centromere copies) was identified. Concerning hepatocellular carcinoma, ESR-1 gene amplification was not confirmed in any case. In contrast, three ($n = 3$) cases demonstrated absence of one allele (ESR-1/CEN 6 ratio <0.8). Furthermore, in thirteen ($n = 13$) tissue cores chromosome 6 polysomy was identified. Colon adenocarcinoma FISH analysis identified only five ($n = 5$) cases characterized by chromosome 6 polysomy, but not ESR-1 gene amplification or absence of alleles. Concerning the overall protein expression, ER-a overexpression was associated strongly to ESR1 gene copies only in breast carcinomas ($P = 0.036$), but not in hepatocellular carcinoma and colon carcinoma in $P = 0.42$, $P = 0.79$), respectively. In breast carcinoma cases, all high gene amplified cores demonstrated ER-a strong expression, but not vice versa.

Discussion

Understanding the genomic instability due to chromosome and gene numerical and structural rearrangements in human solid tumours is essential for developing and applying specific targeted therapeutic agents. Extensive molecular analyses in breast cancer tissues have already confirmed the crucial role of HER2/neu oncogene overexpression via gene amplification and also ER-a/PR expression for estimating the response rates to trastuzumab (monoclonal antibody) and tamoxifen/raloxifene therapy, respectively [16, 17]. Concerning ER-a, ESR-1 gene amplification also regulates its overexpression in a subset of breast cancer cases. It is well known that increased gene copy number leads to an aberrant protein expression in cancerous cells securing their growth and ''immortalization''. HER2/neu, ESR-1 and also other oncogenes, such as CCDN1 and EGFR demonstrate a specific type of gene amplification in solid tumours called double minute chromosomes (multiple gains, isolated or in cluster form) [18] .

In the current study we analyzed a significant number of tissue specimens including breast, hepatocellular and colon carcinomas at the protein expression (ER-a) and DNA level (ESR-1), respectively. We observed that ESR-1 gene amplification characterizes only breast cancer and strongly correlates with ER-a protein overexpression. Although ESR-1 gene amplification seem to be a very important molecular event for handling patients with breast cancer based on targeted therapeutic strategies (i.e tamoxifen), there is an increasing skepticism about the frequency of true amplification in breast cancer [19, 20]. In our study, ESR-1 high gene amplification was detected in a limited subset of the examined cases that overexpressed the marker. Interestingly, another subset of cases characterized by FISH as low gene amplification-based on well established and accepted criteria-overexpressed also the protein [14]. Furthermore, ER-a staining pattern in colon and hepatocellular carcinoma was different compared to breast carcinoma cases. A combined nuclear/perinuclear cytoplasmic stain was observed in those cases. This ER-a immunolocalization has already been identified in other tissues, such as in human fetal testis and epididymis (perinuclear staining of Leydig cells), but the biological significance remain under investigation [21].

Concerning hepatocellular carcinoma analysis, sporadic absence of one allele was identified. Furthermore, analyzing colon adenocarcinoma cases, we failed to detect pure gene numerical imbalances (amplification or allelic absences). In contrast, those malignancies demonstrated chromosome 6 polysomy (centromere gains). There are very limited data regarding the ESR-1 numerical imbalances in hepatocellular and colon adenocarcinoma. Some studies have detected a variety of polymorphisms related to HCC risk among chronic hepatitis B virus carriers [22, 23]. The majority of the studies based on FISH or Comparative Genomic Hybridization (CGH) have identified chromosome 6 aneuploidy and gains or deletions in specific bands on p or q regions that affect the biological behavior of the neoplasm [24–26]. Furthermore, a variant form of estrogen receptor alpha transcript (ER) has been described in hepatocellular carcinoma (HCC). It is derived by an exon 5-deleted transcript (vER), which lacks the hormone-binding domain of the receptor but, being intact in the DNA-binding domain, maintains constitutive transcriptional activity. HCCs presenting vER have an extremely aggressive clinical course and are unresponsive to the antiestrogen tamoxifen [23]. Interestingly, a study based on ESR-1 m- RNA analysis showed that although the majority of the examined HCC tissue samples expressed ER mRNA, the same samples were found to be negative for ER protein by IHC [27]. In our study, a significant subset of HCCs expressed ER-a protein at a low or negative rate. Furthermore, overall expression demonstrated no association with the sporadic numerical allelic imbalances that detected by FISH application. Lack of numerical imbalances regarding ESR-1 gene characterizes also colon adenocarcinoma combined with low or negative expression levels. Despite this, sporadic chromosome 6 gains are observed in cancerous colon tissues but chromosome 8 and 20 polysomy are still the main gross genetic events in this type of cancer [28]. Colon adenocarcinomas demonstrated also low and negative ER-a expression levels without correlation with ESR-1/CEN gains.

In conclusion, this study identified ESR-1 numerical imbalances (amplification, allelic absences) in breast and hepatocellular carcinomas, but not in colon adenocarcinoma. Because ESR-1 gene amplification combined with ER-a overexpression is the basis for hormone targeted therapeutic strategies in breast cancer correlated also with high rates of response in those patients, lack of this deregulation mechanism maybe is a negative factor for applying them in HCCs and CAs. Furthermore, several basic research studies are necessary for concluding if HCC and CA are hormone-depended malignancies or not.

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