Modern Pathology (2009) 22, 191–196

www.modernpathology.org

Estrogen receptor gene amplification occurs rarely in ovarian cancer

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Amplification of the gene encoding estrogen receptor-α occurs in about 20% of breast cancers and is an important mechanism for estrogen receptor overexpression in this tumor type. In ovarian cancer, overexpression of estrogen receptor protein has been described in more than two thirds of cases. To study a potential role of estrogen receptor-α gene amplification for estrogen receptor overexpression in ovarian cancer, a tumor tissue microarray containing 428 ovarian cancers was analyzed by fluorescence *in situ* hybridization for estrogen receptor-α gene amplification and immunohistochemistry for estrogen receptor expression. The estrogen receptor-α gene status was successfully determined in 243 of 428 arrayed cancers. Estrogen receptor gene amplification was found in 5 of 243 (2%) of tumors. Amplification levels were usually low, with 4–8 estrogen receptor-α gene copies. However, one case had a high-level amplification, with more than 30 estrogen receptor-α gene copies. All five amplified tumors were estrogen receptor positive, with 3 of 5 tumors showing highest (Allred score, 7–8) estrogen receptor levels. The data demonstrate that estrogen receptor-α amplification occurs only rarely in ovarian cancer.
Modern Pathology (2009) **22,** 191–196; doi:10.1038/modpathol.2008.130; published online 8 August 2008

Keywords: ovarian cancers; estrogen receptor-α gene; estrogen receptors; fluorescence *in situ* hybridization; immunohistochemistry

Worldwide, ovarian cancer is the fifth most frequent malignant tumor in women and the most common cause of death among cancers of the reproductive system.[1] Prognosis is generally poor as these cancers are often detected in late stage. The median overall survival in these patients is 24–38 months after diagnosis.[2]

Treatment options include surgical removal of the tumor mass with a maximal reduction of the peritoneal cancer mass in case of local tumor extension. In addition, topical and systemic cytotoxic therapy is applied. Ovarian cancer belongs to the group of cancers with frequent expression of steroid hormone receptors. Depending on the study estrogen receptor expression has been reported in 25–86% of ovarian cancers with the highest percentages reported in endometroid and serous subtypes.[3–16] Accordingly, endocrine therapy is a recognized option in the treatment of chemoresistant ovarian cancer after the failure of first- and second-line therapies. However, not all estrogen receptor-positive ovarian cancers respond to antiestrogen therapy, and it was suggested that it might be because of the facts that most of the studies have been retrospective, small in size without adequate selection of the patients and generally used hormonal therapy as a last-line therapy for the refractory or resistant ovarian cancers. Moreover, concerning tamoxifen, it has not been definitely clarified whether it only acts as a pure estrogen antagonist in ovarian tissue, or it has also an agonist effects.[17–21]

In breast cancer, we had recently described estrogen receptor-α (*ESR1*) gene amplification as a frequent mechanism for estrogen receptor overexpression. More than 20% of breast cancers showed *ESR1* gene amplification and more than 15% additional cases low-level *ESR1* gene copy number gains.[22] Preliminary data also suggested that *ESR1* amplified breast cancers may exhibit a high responsiveness to tamoxifen. To determine, whether *ESR1* amplifications also occur in ovarian cancer, we analyzed a set of more than 428 primary ovarian cancers for *ESR1* gene amplification. The results of this study suggest that *ESR1* amplification is a

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Received 16 May 2008; revised and accepted 30 June 2008; published online 8 August 2008

Table 1 Association between histopathological data of ovarian cancers and estrogen receptor protein expression and *ESR1* amplification

		Estrogen receptor immunohistochemistry result (ALLRED score) (%)							*ESR1 FISH results*		
		On TMA	*Analyzed (n)*	*0–2*	*3–4*	*5–6*	*7–8*	P-*value*	*Analyzed (n)*	*Amplification (%)*	P-*value*
	All cancers	428	384	62.8	11.2	16.7	9.4		243	2.1	
	Serous carcinoma	172	158	48.7	16.5	22.8	12.0	0.0071[a]	105	1.9	n.s.[a]
	Mucinous carcinoma	76	69	84.1	2.9	7.2	5.8	0.0306[b]	40	2.5	n.s.[b]
	Endometroid	85	80	55.0	13.8	21.3	10.0	n.s.[c]	44	4.5	n.s.[c]
	Mullerian mixed cancer	15	14	100.0	0.0	0.0	0.0	n.a.	7	0.0	n.a.
Histology	Clear cell cancer	24	24	100.0	0.0	0.0	0.0	n.a.	13	0.0	n.a.
	Malignant Brenner tumor	5	4	50.0	0.0	50.0	0.0	n.a.	3	0.0	n.a.
	SQCC	1	1	100.0	0.0	0.0	0.0	n.a.	1	0.0	n.a.
	Sex cord-stromal tumors	10	10	60.0	10.0	0.0	30.0	n.a.	8	0.0	n.a.
	Yolk sack tumor	4	4	75.0	25.0	0.0	0.0	n.a.	2	0.0	n.a.
	Undifferentiated carcinoma	15	15	46.7	13.3	26.7	13.3	n.a.	10	0.0	n.a.
	Other rare types	5	5	100.0	0.0	0.0	0.0	n.a.	10	0.0	n.a.
	pT1	58	54	75.9	5.6	13.0	5.7		25	0.0	
pT stage	pT2	36	32	78.1	6.3	6.3	9.4	0.1343	19	0.0	n.s.
	pT3	99	88	58.0	15.9	18.2	8.0		58	1.7	
	G1	81	71	71.8	5.6	14.1	8.5		33	0.0	
Silverberg grade	G2	91	82	72.0	11.0	12.2	4.9	0.038	52	0.0	n.s.
	G3	91	85	51.8	20.0	21.2	7.1		55	1.8	

[a]Serous versus mucinous, [b]mucinous versus endometroid, [c]serous versus endometroid.
SQCC, squamous cell carcinoma.

mechanism for estrogen receptor overexpression only in a very small subset of ovarian cancers.

Materials and methods

Tissues

A tumor tissue microarray constructed from primary tumors of 428 ovarian cancer patients was used for this study. The median patient age was 58.1 (range, 24–84) years. Raw survival data were either obtained from the cancer registry of the University of Basel, University Hamburg or collected from the patients attending physicians. The mean follow-up time was 41.85 months (range, 1–210). Formalin-fixed (neutral-buffered aqueous 4% solution), paraffin-embedded tumor material was utilized. The pathologic stage was obtained from the primary pathology reports. All slides from all tumors were reviewed by one pathologist (HM) to define the histological grade and the histological tumor type. The composition of the tumor tissue microarray is described in detail in Table 1.

Fluorescence *In Situ* Hybridization

Tumor tissue microarray sections were treated according to the Paraffin Pretreatment Reagent Kit protocol (Vysis, Downers Grove, IL, USA) before hybridization. Fluorescence *in situ* hybridization (FISH) was performed with a digoxigenated BAC probe (BAC RP11-450E24, RZPD, Germany) containing a part of the *ESR1* gene and a Spectrum-Orange-labeled chromosome 6 centromeric probe as a reference (purchased from Vysis). Hybridization and post-hybridization washes were according to the 'LSI procedure' (Vysis). Probe visualization using fluorescent isothiocyanate-conjugated sheep anti-digoxigenin (Roche Diagnostics, Rotkreuz, Switzerland) was as described.[23] Slides were counterstained with 125 ng/ml 4′, 6-diamino-2-phenylindole in an antifade solution. Hybridization and post-hybridization washes were according to the 'LSI procedure' (Vysis). Slides were then counterstained with 125 ng/ml 4′,6-diamino-2-phenylindole in an antifade solution. The number of fluorescence signals was estimated by an experienced person (FH) in each tissue spot for the centromere 6 and the *ESR1* gene probes. *ESR1* alterations were defined based on the ratio of gene copy numbers of *ESR1* and centromere 6. Tissues with at least twofold more *ESR1* than centromere 6 copies (ratio ≥ 2.0) were considered '*ESR1* amplified'. Tissues with more *ESR1* than centromere 6 copies not reaching the criteria for amplification were considered '*ESR1* gained' (ratio > 1.0 but < 2.0). All other analyzable tissues (ratio 1.0) were considered '*ESR1* normal'.

Immunohistochemistry

Immunohistochemical detection of estrogen receptor protein was performed using a monoclonal antibody (DAKO no. M7047, clone 1D5). In brief, 4 μm tumor tissue microarray slides were deparaffinized in Xylol prior to heat induced antigen retrieval using DAKO's antigen retrieval solution pH9 (DAKO no. S2368). The primary antibody was diluted 1:50 and incubated for 30 min at room temperature. The primary antibody was omitted for negative control. All spots were analyzed by one pathologist (RI). Immunohistochemical scoring was performed according to the Allred score.[24] In brief, estrogen receptor staining intensity was recorded in a 4-step scale (0–3) and the fraction of positive tumor cells in a 5-step (1–5) scale. Combination of both parameters results in an 8-step score, where all samples with score >2 are regarded as estrogen receptor positive.

Statistical Analysis

Contingency table analysis and χ^2-tests were used to study the relationship between clinicopathological parameters of the analyzed tissues and estrogen receptor expression levels. Kaplan–Meier plots and log-rank tests were employed to analyze the relationship between estrogen receptor expression status and patient survival.

Results

ER Expression

Immunohistochemical estrogen receptor analysis was successful in 384 of 428 (89, 7%) arrayed samples. Analysis failure was due to lack of tumor cells in tissue spots ($n=19$, 4.4%) or missing tissue spots ($n=24$, 5.6%). More than one-third (148 of 384, 37.2%) of tumors showed at least weak estrogen receptor expression. Strongest staining (score 7–8 according to Allred) was found in 36 of 384 (9.4%) of samples, and was linked to high-grade cancers ($P=0.038$). Estrogen receptor expression was unrelated to patient prognosis ($P=0.2491$, Figure 1). Examples of immunohistochemically positive and negative tumors are shown in Figure 2. All immunohistochemistry results are summarized in Table 1.

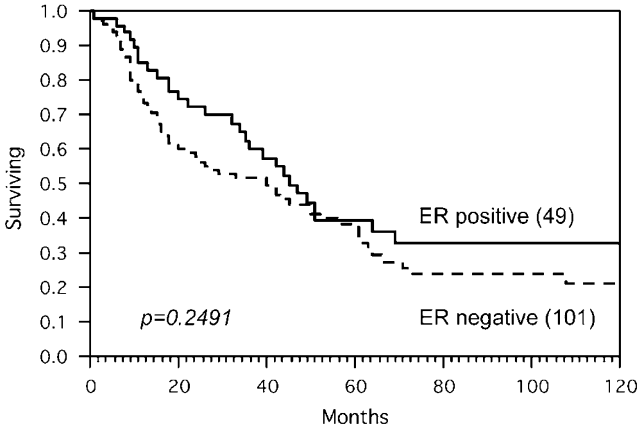

Figure 1 Kaplan–Meier analysis of estrogen receptor (ER) positive and negative ovarian cancers.

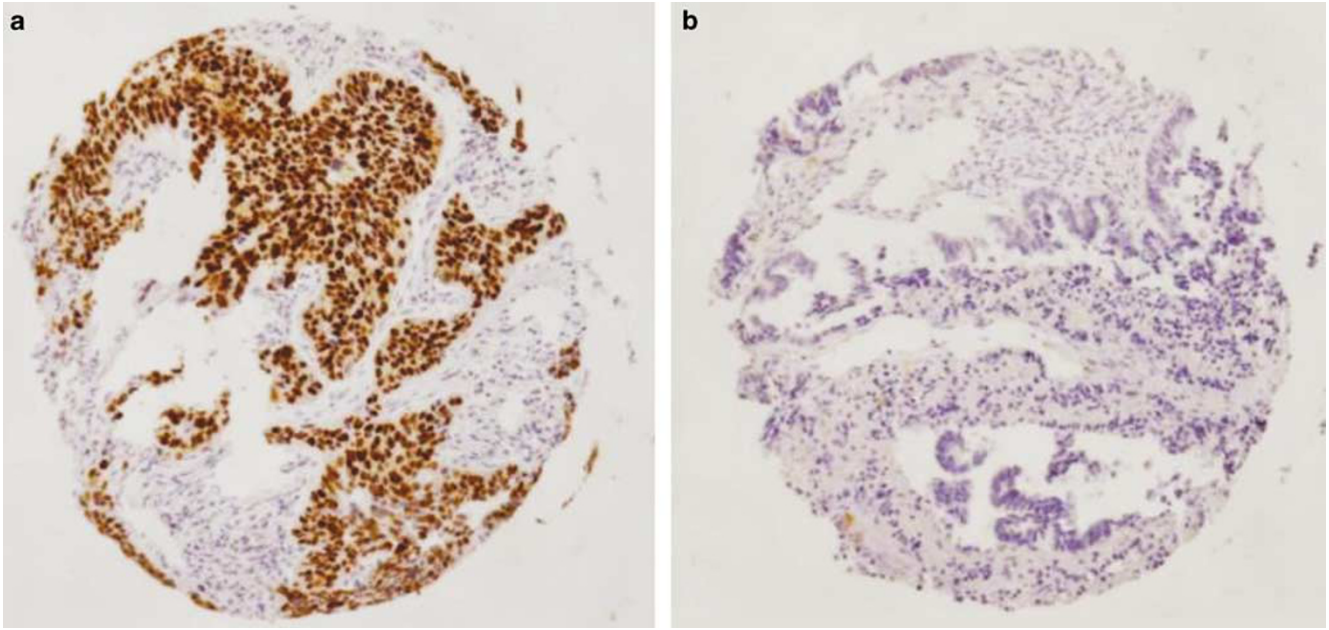

Figure 2 Examples of estrogen receptor positive (**a**) and negative (**b**) ovarian cancers. Immunohistochemistry, $\times 100$ magnifications.

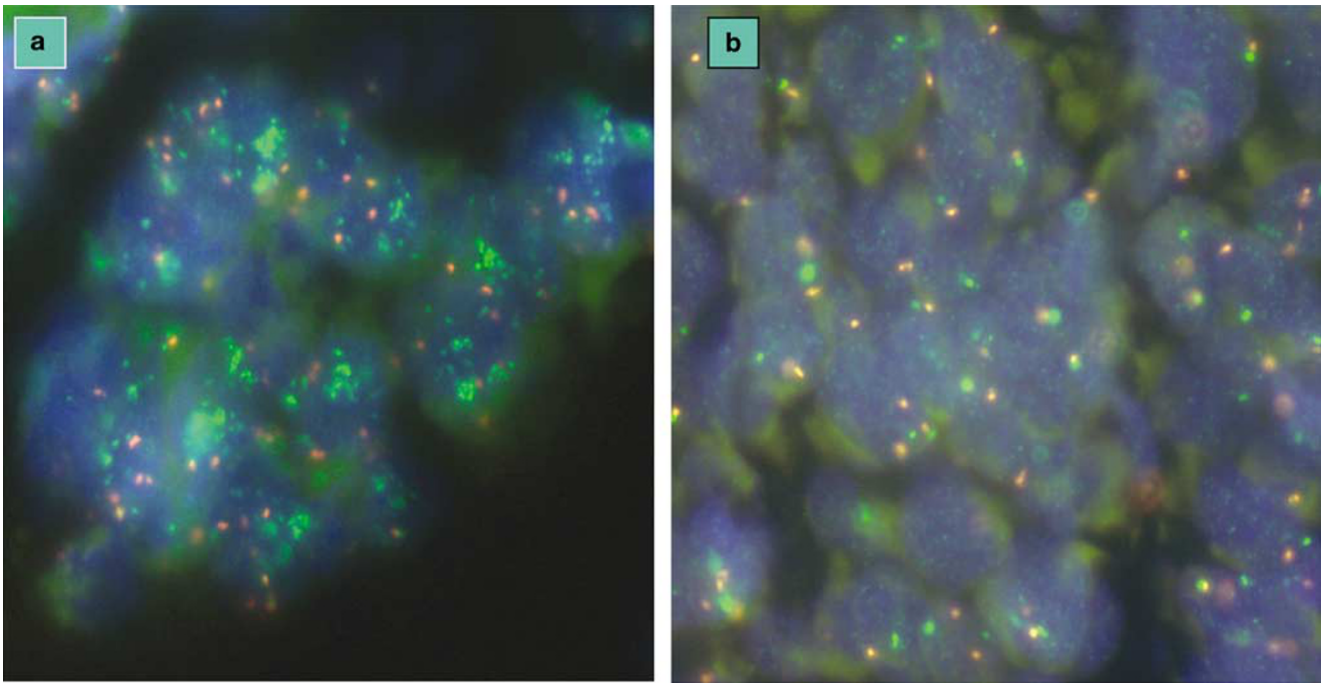

Figure 3 Examples of ovarian cancers with *ESR1* amplification (**a**) and with normal *ESR1* copy numbers (**b**). Red signals indicate copy number of centromere 6; green signals indicate *ESR1* copy numbers. FISH analysis, ×630 magnifications.

ESR1 Amplification

ESR1 FISH analysis was successful in 243 of 428 arrayed tissue samples. Missing results were either due to missing tissue samples on the tumor tissue microarray ($n=80$) or lack of interpretable FISH signals ($n=105$). *ESR1* amplification (ratio *ESR1*/centromere $6 \geq 2.0$) was found in 5 of 243 (2.1%) tumors. Amplifications were usually low level with 4–8 FISH signals. One sample had a high-level amplification (>10 signals; Figure 3). Examples of *ESR1* amplified and non-amplified tumors are shown in Figure 3. *ESR1* amplification was unrelated to histopathological parameters including histological subtype, tumor stage, and grade. No survival analysis was performed because of the small number of cases with *ESR1* amplification.

All five tumors with *ESR1* amplification were variably positive for estrogen receptors protein expression with strong positivity in three out of five cases.

Discussion

The results of this study show that *ESR1* amplification is rare in ovarian cancers (2.1%). More than one-third of ovarian tumors showed immunohistochemically detectable estrogen receptor protein expression, most abundant in serous and endometroid subtypes. This is in line with previous studies done on the classical paraffin blocks. The good concordance between our data and previous studies demonstrates the representation of our tumor tissue microarray data obtained on a 0.6 mm tissue spot per tumor and enhances the results of other studies used in this method.

A small subset of *ESR1* amplified estrogen receptor-positive cases was indeed found in ovarian cancers. In comparison, some other genes showed higher rates of amplifications in these cancers. For example, the amplification of *ERBB2* ranges (0–66%),[25,26] *EGFR* (3.65–12%),[27,28] *CCND1* (0–19%),[29–31] *C-MYC* up to 54.5,[25,32,33] and *KRAS* (31%).[33]

The significant frequency of estrogen receptor positivity in ovarian cancers had prompted treatment efforts using hormonal therapy early on. In addition their relatively little toxicity was another provoking factor to continue going on to achieve more advance in this therapeutic field. Monotherapy studies using tamoxifen, aromatase inhibitors, and GnRH analogues had yielded variable results with objective response rates ranging between 0 and 56%.[17,19–21,34–38] Combinatorial treatment regimens combining tamoxifen and goserelin or tamoxifen and Gefitinib had obtained results with objective response rates of up to 11.5%.[39,40] Few of these studies had selected patients based on the immunohistochemically determined estrogen receptor status. It is therefore unclear, whether the estrogen receptor expression level has any impact on the likelihood of response, or this just reflects the lack of establishment of well-organized treatment strategy in previously heavily treated patients and who in significant part already suffered from advanced disease.

The role of estrogen receptor expression for response prediction to anti-hormonal drugs has been much better studied in breast cancer, where a strong association between estrogen receptor positivity and response to anti-hormonal drugs is well established. However, also in breast cancer, not all estrogen receptor-positive cancers respond to tamoxifen and related drugs. In a recent study, we had found that *ESR1* amplification may be strongly predict tamoxifen response among estrogen receptor-positive breast cancers. More than 20% of breast cancers had amplified or at least elevated *ESR1* copy number. Possible explanations for the predictive effect of ESR1 amplification could be a particularly high expression of amplified as compared to non-amplified cancers. Alternatively, it could be speculated, that *ESR1* amplified are more dependent on the estrogen receptor pathway than other tumors that express estrogen receptors together with many other growth receptors. If this latter hypothesis was true, visualization of *ESR1* amplification would pinpoint toward an 'Achilles tendon' of a tumor that could be most successfully targeted.

The frequency of *ESR1* amplified ovarian cancers (2.1%) is much lower than that in breast cancer. Interestingly, this fraction somehow parallels the percentage of ovarian cancers reported to show strong responses to hormonal therapies. For example, in retrospective, analysis was conducted of patients who received tamoxifen at a dose 20 mg twice daily for the treatment of advanced epithelial ovarian cancer, Karagol *et al*[41] found that out of 29 eligible patients included in the study, there were 1 (3%) complete response, 2 (7%) partial response, 6 (21%) stable disease, and 20 (69%) progressive disease. Papadimitriou *et al*[35] have studied response rate in 27 patients treated with letrozole at a dose of 2.5 mg once a day. Patients with measurable or evaluable disease ($n=21$) and those with only increasing CA-125 serum levels ($n=6$) were eligible. Among the 21 patients with measurable or evaluable disease, 1 complete response (5%) and 2 partial responses were observed (10%) for an objective response rate of 15%. Other studies, in which the combined regiment had been implicated, patients were given oral tamoxifen 20 mg twice daily on a continuous basis and subcutaneous goserelin 3.6 mg once a month until disease progression. In total, 26 patients entered this study, of which 17 had platinum-resistant disease, using the definition of endocrine response that included patients with stable disease of 6 months or greater, the overall response rate (clinical benefit rate) was 50%. This included one complete response (3.8%), two partial responses (7.7%), and 10 patients with stable disease (38.5%).[39]

In summary, *ESR1* amplification is an uncommon mechanism for estrogen receptor overexpression in ovarian cancer occurring in about 2.1% of the total number of ovarian cancers. In general, this frequency parallels the fraction of ovarian cancers reported to show complete response to antiestrogenic therapies. Given the strong predictive power of *ESR1* amplification for response to tamoxifen in breast cancer, an evaluation of such treatments in *ESR1* amplified ovarian cancers appears justified.

Acknowledgements

We are grateful to Ms Michaela Härtling, Ms Sandra Schmidt, Ms Silvia Schnöger, and Mr Sascha Eghtessadi for excellent technical assistance in immunohistochemistry and FISH analysis, and to Ms Martina Mirlacher for tumor tissue microarray making.

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