Immunohistochemical Analysis of WT1, EGFR, E-cadherin, beta-catenin and p53 in 43 Moroccan Epithelial Ovarian Tumours

Mariam Amrani1, Lorenzo Memeo2, Habiba Kadiri3, Hind Charhi4, Mohamed Alaoui Belabbes1, Mahesh M. Mansukhani3

1Department of Pathology, Institute National d’Oncologie, Rabat, Morocco
2Department of Experimental Oncology, Mediterranean Institute of Oncology, Catania, Italy
3Department of Pathology, Columbia University Medical Center, New York, NY, USA
m.amrani@um5s.net.ma

Abstract- Aim of the study: Immunohistochemical evaluation of WT1, E-cadherin, beta-catenin, EGFR and p53 on Tissue MicroArray (TMA) of 43 Moroccan benign, borderline and invasive epithelial ovarian tumours. Materials and methods: All 43 cases were collected from the pathology department of the Institut National d’Oncologie in Rabat, Morocco, and comprised 34 carcinomas, 4 borderline serous and mucinous tumours and 5 benign tumours. Patients were between 20 and 74 years old with a mean age of 50 years. TMAs and the IHC study were supported by a grant from the IAAE (International Agency of Atomic Energy) and prepared in the pathology department of Columbia University in New York. 3 cores were selected from each case, and the peroxysdase-anti peroxysdase technique was used for the study of the different markers (DAKO Cyтовision, Carpinteria CA). Results: 23.25% of the cases (10/43) were WT1 positive and were serous tumours (including one poorly differentiated adenocarcinoma). 72% of the cases (31/43) showed reduced (19/43) or no (12/43) membranous expression of E-cadherin, and all the tumours showed reduced membranous expression with cytoplasmic expression (5/43) or no expression (38/43) of beta-catenin. p53 overexpression (13/43) was exclusively observed in 58% (11/19) of the serous carcinomas and 2/3 poorly to moderately differentiated adenocarcinomas, of which 9/13 were EGFR + and 6/13 were E-cadherin +, 70% of the cases (30/43) showed EGFR membrane staining, and 2 cases were not interpretable. Conclusion: TMA is a feasible tool to study a large number of cases allowing comparative analysis of the expression of different biomarkers. To our knowledge, this is the first study of 5 biomarkers to be done on TMAs from 43 moroccan benign, borderline and invasive epithelial ovarian tumour samples. This would allow for larger studies with the aim of analyzing the significance of these biological markers and their impact in clinical trials.

Keywords- Ovarian Epithelial Tumours; Tissue Microarray; EGFR Protein Marker; Suppressor Protein Markers; Wnt Protein Markers

I. INTRODUCTION

A tissue microarray allows for an analysis of hundreds of specimens with one slide instead of incubating and analyzing samples one slide at a time. All the histochemical and molecular detection techniques that can be used with regular sections can also be used with tissue microarrays. Typical applications include immunohistochemical detection of protein expression in clinical tissue specimens.

To introduce this technique in our institution, we chose to apply it to a set of Moroccan epithelial ovarian tumours and study the immunohistochemical expression of 5 biomarkers.

Ovarian cancers are represented in 90% of the cases by surface epithelial carcinomas comprising different histological phenotypes with the serous carcinomas being the most frequent. Because of the lack of early symptoms, ovarian carcinomas are mostly advanced-stage at diagnosis and, therefore, associated with a high mortality rate and a development of drug resistance. Many biological events are thought to play a role in the carcinogenesis of these tumours, making it difficult to give accurate prognostic information for all ovarian cancer patients. Among the multiple biomarkers reported in the literature, we chose to study the protein expression of 2 suppressor genes, WT1 and p53, EGFR, and 2 proteins of the Wnt signaling pathway, E-cadherin and beta-catenin.

II. MATERIAL AND METHODS

All 43 cases were collected from the pathology department of the Institut National d’Oncologie in Rabat, Morocco, and the histologic types were as shown in Table 1.

Patients were between 20 and 74 years old with a mean age of 50 years.

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DOI:10.5963/BER0301002
Three (3) cores were selected from each case, and 2 receiver paraffin blocks were prepared with the Beecher manual arrayer.

Two slides were stained with the routine haematoxylin-eosin stain for morphologic analysis and 10 slides were used for immunohistochemical analysis (Fig. 1) instead of 215 slides if each case had to be represented separately on a slide.

![Image of TMA slides](image)

**Fig. 1** The 12 TMA slides representing the 43 ovarian tumours.

Immunohistochemical analysis was performed with the peroxydase-anti peroxydase technique, and the 5 biomarkers studied were as shown in Table 2. The incubation time was 40 minutes for each biomarker.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution</th>
<th>Treatment</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1</td>
<td>1/45</td>
<td>EDTA pH = 9</td>
<td>Cell Marque</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>1/100</td>
<td>citrate pH = 6.</td>
<td>Dako</td>
</tr>
<tr>
<td>Beta-catenin</td>
<td>1/100</td>
<td>citrate pH = 6.</td>
<td>Dako</td>
</tr>
<tr>
<td>p53</td>
<td>1/45</td>
<td>citrate pH = 6.</td>
<td>Biogenex</td>
</tr>
<tr>
<td>EGFR</td>
<td>1/45</td>
<td>Proteinase K</td>
<td>Zymed</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution</th>
<th>Treatment</th>
<th>Company</th>
</tr>
</thead>
</table>

Scoring of biomarkers staining Figure 2:

E-cadherin and beta-catenin: 3 categories according to the percentage of positive cells (0-10% negative, 11-50%, more than 50%), staining intensity (0 or less than 10% negative; staining observed under high power field 400: 1+ weak; staining...
observed even under low power field 50: 3+ strong; in between: 2+ intermediate), and structures stained (C: cytoplasm, C/M: cytoplasm and membrane, C/M/N: cytoplasm and/or membrane and nucleus). ImmunoHistoChemical Scoring (IHCS) = staining intensity x percentage of positive cells. IHCS < or equal 100 was considered reduced expression.

EGFR: Positive stain when at least 10% of the cells were stained with distinct staining of the cytoplasmic membrane (sometimes associated with cytoplasm staining) and were often unevenly distributed within the tumour; some cases show only focal positivity in different areas of the tumour.

WT1: Regardless of nucleus staining intensity: +: 1-10%; ++: 11-50%; +++: more than 50%.

P53: Nuclear immunostaining for p53 was scored negative if less than 10% tumour cells showed nuclear staining; + for 30—60%; ++ for 60—90%, and +++ if more than 90%. Staining intensity was scored weak, moderate or strong.

![Images of stained tissues](image1.png) ![Images of stained tissues](image2.png) ![Images of stained tissues](image3.png) ![Images of stained tissues](image4.png)

Fig. 2 WT1 strong nuclear staining [1], p53 moderate nuclear staining [2], EGFR strong membranous and cytoplasmic staining [3], E-cadherin moderate membranous staining

### III. RESULTS

23.25% of the cases (10/43) were WT1 positive and were serous tumours (one of which was a poorly differentiated adenocarcinoma). 30.23% p53 overexpression (13/43) was exclusively observed in 58% (11/19) of the serous carcinomas and 2/3 poorly to moderately differentiated adenocarcinomas, of which 9/13 were EGFR +. On the other hand, 6/13 p53 positive cases were also E-cadherin +.

72% of the cases (31/43) showed reduced membranous expression (19/43) or no membranous expression (12/43) of E-cadherin, and all the tumours showed reduced membranous expression with cytoplasmic expression (5/43) or no expression (38/43) of beta-catenin. 70% of the cases (30/43) showed EGFR membranous staining, and 2 cases were not interpretable; 47.36% (9/19) of the serous carcinomas and the 1 borderline serous tumour were EGFR negative (Table 3).

<table>
<thead>
<tr>
<th>Table 3 Results of Tumour Expression of the 5 Biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT1</strong></td>
</tr>
<tr>
<td>34 carcinomas</td>
</tr>
<tr>
<td>7 mucinous</td>
</tr>
<tr>
<td>3 endometriode</td>
</tr>
<tr>
<td>2 CCC</td>
</tr>
<tr>
<td>3 ADK P to M diff</td>
</tr>
<tr>
<td>4 borderline tumours</td>
</tr>
<tr>
<td>1 serous</td>
</tr>
<tr>
<td>DOI:10.5963/BER0301002</td>
</tr>
</tbody>
</table>
IV. DISCUSSION

A. WT1:

WT1 positivity in our study is in agreement with the literature [1] [2] and may be useful in differentiating ovarian serous carcinomas, especially when the tumour is poorly differentiated, such as from a breast carcinoma metastatic to the ovary (WT1 negative) [3].

This is of particular interest when searching for an ovarian (WT1++) or endometrial (WT1+/−) origin of a disseminated serous carcinoma since endometrial serous carcinomas respond worse to platinum-based chemotherapy [4].

Due to the specificity and high sensitivity of WT1 [5] for metastatic carcinoma of ovarian primary, accurate diagnosis of small round cells tumours of the ovary can be achieved with an immunohistochemical panel, including WT1 and TTF1, Inhibin, Cytokeratin, leucocyte common antigen and endocrine specific antibodies [6].

A recent study [7] reported WT1 positive immunostaining of ovarian endometrioid carcinomas in correlation with histologic grade and depending upon whether the tumour arose from ectopic endometrial tissue or from the normally WT1− positive ovarian surface epithelium. Further studies are therefore needed for endometrioid carcinomas since we only had 3 cases.

WT1 was also found to be highly specific for high grade serous carcinomas [8] in the differential diagnosis with clear cell carcinomas, which do not respond well to conventional chemotherapy. The diagnostic panel, recommended by Köbel et al. in their study, included WT1 and oestrogen receptor and hepatocyte nuclear factor (HNF)-1beta, the latter being the most specific for clear cell carcinomas [9].

Recently, Liliac et al. [10] recommended the association of PAX8 for the confirmation of an ovarian primary and WT1 for the phenotyping, especially in the case of serous ovarian carcinomas [10].

In a multivariate analysis, WT1 was not found to be an independent marker of survival but is considered a diagnostic marker of great utility that highlights the serous component of mixed ovarian carcinomas, since their prognosis is thought to be dependent on the presence of the serous differentiation [11].

B. p53:

The review of Cécile Le Page et al. [11] points out the discrepancies found in the literature about the prognostic value of p53 in ovarian cancer, especially in clinical studies using paclitaxel-based chemotherapy treatment. Since immunohistochemistry is routinely used to evaluate p53 status, one of the possible reasons for these discrepancies is likely to be inherent to technical issues and the different antibodies used.

In the same review [11], Bax expression (a pro-apoptotic Bcl2 family member) was found to correlate with apoptosis and a patient’s survival in ovarian cancer, especially in the early stage in a p53 mutant subgroup but not in the p53 wild type subgroup; Bax expression was also found to correlate with complete remission but not when patients were separated into the two p53 subgroups.

In the Tachibana et al. article [12], p53 is reported as a poor prognostic marker in ovarian carcinomas and comcomitant expression of p53 and EGFR, exclusively observed in our study in serous carcinomas and poorly to moderately differentiated adenocarcinomas, and define the worst prognostic group compared to cases that are either p53 or EGFR + (intermediate-risk

<table>
<thead>
<tr>
<th>3 mucinous</th>
<th>1 NE 2/3 RE</th>
<th>NE</th>
<th>2/3 +</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 benign tumours</td>
<td>NE</td>
<td>60% (3/5)</td>
<td>100% (5/5)</td>
</tr>
<tr>
<td>3 serous</td>
<td>2/3 +</td>
<td>1 NE 2/3 RE</td>
<td>2 NE 1/3 RE</td>
</tr>
<tr>
<td>1 mucinous</td>
<td>1/1 N</td>
<td>NE</td>
<td>1/1 MS</td>
</tr>
<tr>
<td>1 Brenner</td>
<td>1/1 N</td>
<td>NE</td>
<td>1/1 MS</td>
</tr>
<tr>
<td>Total</td>
<td>10/43</td>
<td>13/43</td>
<td>12 NE 19 RE</td>
</tr>
</tbody>
</table>

CCC: Clear Cell carcinoma
ADK P to M diff: Adenocarcinoma poorly to moderately differentiated
NE: No Expression
RE: Reduced Expression
OE: Over Expression
N: Normal
MS: Membranous Staining

DOI:10.5963/BER0301002
group) or when both the prognostic markers are negative (low-risk group) [13]. We also observed coexpression of p53 and WT1 in 6 carcinomas. Because WT1 might “rescue” cells from p53-induced apoptosis [1], this might have relevance to chemotherapy as the efficacy of chemotherapy relies on apoptosis.

p53 overexpression was also found to characterize a set of high grade endometrioide ovarian carcinoma compared with a low-grade group that was associated with beta-catenin and KRAS mutations [14].

Therefore, it might be more significant to evaluate p53 in association with other markers than p53 by itself.

C. E-cadherin:

E-cadherin membranous staining has been reported as a possible independent marker of good prognosis in ovarian carcinomas [15]. However, concomitant expression of p53, as found in our study (6/13 p53 positive cases were also E-cadherin +), might correlate with a worse prognosis indicating that a combination of two or more independent factors may yield an improved overall prognostic index.

On the other hand, reduced membranous E-cadherin expression is correlated with a bad prognosis in gastric, breast and prostate carcinomas. Reduced or no expression was observed in 70.5% of the carcinomas in our study. Loss of E-cadherin expression was found to promote tumor progression and metastasis through the up-regulation of alpha 5-integrin which might be a therapeutic target in a subgroup of ovarian cancers [16].

D. Beta-catenin:

As for beta-catenin membranous expression, all our 43 tumours showed absence or reduced membranous expression, a change which is correlated with a high invasive potential [17] and an early recurrence of ovarian endometrioide carcinoma [18].

Other authors found a strong cytoplasmic and sometimes nuclear expression, especially in endometrioide carcinomas [15]: indicating nuclear staining to be a good prognostic marker with intermediate overall survival and late disease recurrence [18] whereas exclusive membrane staining identifies a sub-group of endometrioide carcinoma with bad prognosis correlating with relapse and death. Our samples included only 3 such tumours and further investigation with more samples is needed to determine whether this indicates a different pathogenic pathway for some histological types of carcinomas.

Beta-catenin has also been found to play a role in differential diagnosis of metastatic colorectal cancer to the ovaries as indicated by 83% of these tumours showing nuclear staining while only 9% of primary ovarian mucinous carcinomas expressed beta-catenin [19].

Stawerski et al. [20] found an increased reduction in the immunoeexpression of both E-cadherin and beta-catenin when considering benign, non metastatic and metastatic serous ovarian carcinomas. They suggest that these markers could help in defining cases with metastatic and infiltrative potential.

We had too few borderline tumors in our group to draw significant conclusions although all of our tumours were either beta-catenin negative or showed reduced membranous expression, which contradicts the results of Davies BR. et coll. [17]: all the benign tumours in his study were beta-catenin positive.

E. EGFR:

EGFR and Her-2 are members of the tyrosine kinase receptor (TKR) family and were found to have little impact on patient outcome if not restricted to specific histologic subtypes of ovarian cancer. Nevertheless, 20% of mucinous carcinomas were found to overexpress Her-2 which might be of therapeutic interest when targeting this TKR [11].

In other studies, EGFR expression is considered a bad prognostic marker in ovarian cancer, leading to tumor proliferation, invasion, metastasis and inhibition of apoptosis with the selection of a group of high risk when associated with p53 coexpression [13]. In our study, 70% of the carcinomas expressed EGFR which is a little higher than the 60% reported in the literature [20] and in contrast to the 12% reported by Henzen-Logmans et al. [22].

However, in recent studies, EGFR overexpression detected by immunohistochemistry was found to less reliably detect EGFR amplifications than FISH, amplifications being more frequent than EGFR mutations in ovarian cancer [23] [24]. This is of great importance since the treatment response to EGFR inhibitors appears to correlate with amplification.

In the clinical trial reported by Gordon A.N. et coll.[25], Erlotinib HCl (erlotinib, Tarceva, OSI-774; OSI Pharmaceuticals, Melville, NY), an inhibitor of EGFR, was used alone in 34 patients exhibiting EGFR positive ovarian cancers refractory to taxane and/or platinum based chemotherapy. There was a partial response in 6% of the cases indicating minimal antitumoural activity when Erlotinib HCl is used in monotherapy. This could be explained by the fact that inhibition of EGFR might restore cell proliferation via the activation of other molecular signaling pathways.

It was also stated that resistance to Paclitaxel might be the result of the activation of cell survival factors via the transient activation of EGFR by the use of Paclitaxel [26].
The authors therefore recommend a combination of EGFR inhibitors with other chemotherapeutic agents.

V. CONCLUSION

In summary, carcinomas in our study show a highly invasive and metastatic profile when taking into account E-cadherin, beta-catenin, p53, WT and EGFR as biomarkers. It would be interesting to determine if EGFR overexpression in our cases is due to amplifications and to study more cases in order to understand the significance of the expression of these markers in benign and borderline tumours.

These results, reported for the first time to our knowledge, in a group of Moroccan patients, would allow for larger studies with the aim of analyzing the correlation of these biological markers with clinicopathologic parameters and their impact in clinical trials.

In our study only 10 IHC slides were analyzed instead of 215 slides if each case was studied with each biomarker on a separate slide. Other advantages of TMA, especially in cancer research, are ease-of-use, standardization, conservation of valuable tissue, analysis of the frequency of a molecular alteration in different tumour types, evaluation of diagnostic and prognostic markers and optimization of antibody staining conditions. However, it should be kept in mind that some pitfalls such as tumour heterogeneity and loss of antigenicity for some antibodies might influence the interpretation of the results, and therefore, quality control guidelines are mandatory.

TMA is therefore a suitable technique especially if correlated with the study of associated genetic alterations which might more precisely yield the possible characteristics of Moroccan patients and allow personalized treatment, at least for the disease stabilization and a better quality of life.

ACKNOWLEDGMENT

The authors would like to warmly thank INO staff (especially Boudaz Khalid and Bathami El Khayati for samples collection), the experimental molecular pathology laboratory staff at Columbia University Medical Center (New York, USA) (especially Lyn Yang and Rina Wu for their excellent technical work) and the International Agency of Atomic Energy, (Vienna, Austria) for financing the study.

REFERENCES


DOI:10.5963/BER0301002


