

# High-grade fimbrial-ovarian carcinomas are unified by altered p53, PTEN and PAX2 expression

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**High-grade endometrioid and serous carcinomas of the ovary and fallopian tube are responsible for the majority of cancer deaths and comprise a spectrum that includes early or localized (tubal intraepithelial carcinoma) and advanced (invasive or metastatic) disease. We subdivided a series of these tumors into three groups, (1) classic serous, (2) mixed serous and endometrioid and (3) endometrioid carcinomas and determined: (1) the frequencies of coexisting tubal intraepithelial carcinoma, (2) frequency of a dominant ovarian mass suggesting an ovarian origin and (3) immuno-localization of WT-1, p53, PTEN, PAX2 and p16<sup>ink4</sup>. All tumors were analyzed for p53 mutations. Thirty six, 25 and 8% of groups 1–3 were associated with tubal intraepithelial carcinoma ( $P=0.09$ ) and 34, 45 and 62% predominated in one ovary ( $P=0.028$ ), respectively. Differences in frequencies of diffuse p53 immunostaining (85–93%), WT-1 (70–98%) and p16<sup>ink4</sup> positivity (69–75%) were not significant for all groups. Greater than 95% reduction in PAX2 and PTEN occurred in 67–75 and 5–12%, respectively; however, PAX2 and PTEN staining intensity, when present, was often heterogeneous, highlighting different tumor populations. PAX2 and PTEN expression were markedly reduced or absent in 12 of 12 and 4 of 12 tubal intraepithelial carcinomas. In summary, high-grade müllerian carcinomas share identical frequencies of altered or reduced expression of p53, PTEN and PAX2, all of which can be appreciated in tubal intraepithelial carcinomas. Because only a subset of these tumors appears to arise in the fallopian tube, attention to expression of these biomarkers in the ovary and other müllerian sites might facilitate the identification of other carcinogenic pathways. PAX2 and PTEN, in addition to p53 and p16<sup>ink4</sup>, comprise a potentially important gene combination in high-grade pelvic carcinogenesis.**

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Malignant pelvic (ovary, fallopian tube and peritoneal) epithelial neoplasms can display a diverse array of histological patterns, including serous,

endometrioid, mucinous and clear cell differentiation. Studies interrogating the molecular profiles and clinical behavior of these different tumors have demonstrated that they can be partitioned into two general pathways of tumor development.<sup>1</sup> The 'type I' pathway is exemplified by the endometrioid, clear cell, mucinous and low-grade serous carcinomas. These tumors are associated with mutations in genes encoding mismatch repair proteins and signaling proteins governing cell proliferation, such as BRAF, KRAS,  $\beta$ -catenin and PTEN, but not mutations in p53.<sup>2–4</sup> Type I tumors are felt to

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evolve in a step-wise manner from known pre-existing conditions such as cortical inclusion cysts, endometriosis, cystadenomas and adenofibromas to borderline tumors to invasive malignancies, and appear to be the prototype for an ovarian-derived carcinogenic sequence.<sup>5</sup> In contrast, high-grade serous carcinomas have been assigned to the 'type II' pathway and the lack of a clear-cut precursor condition has, until recently, prompted the theory that these tumors arise rapidly through p53 mutations, an assumption encouraged by the fact that most of these tumors are discovered at an advanced stage.<sup>6</sup>

Two observations have progressively refined our understanding of high-grade serous carcinomas. The first has been the discovery that the fallopian tube is a site of origin for many of these tumors, highlighted by protocols that extensively sampled the fallopian tube, especially the fimbriated end, for histological evaluation.<sup>7-9</sup> These protocols have revealed that from 35 to 45% of high-grade serous carcinomas are associated with a tubal intraepithelial carcinoma, suggesting an origin from the tube.<sup>10-13</sup> The second observation has been the increasing similarity between high-grade serous and high-grade endometrioid carcinomas, including the finding of p53 mutations in the latter.<sup>14-17</sup> As a consequence, these two subsets of high-grade carcinomas have often been grouped together. However, we have previously shown that the two tumor types are not equivalent in terms of their association with either tubal intraepithelial carcinoma or tumor distribution, with the endometrioid tumors having a lower frequency of the former and a higher frequency of association with a dominant (greater than 2X length in the greatest dimension) ovarian mass.<sup>13</sup> The differences in the frequency of these parameters suggest differences in potential tumor origins for high-grade serous (more frequently tubal) and endometrioid (more frequently ovarian) carcinomas.

The purpose of this study was to determine (1) if high-grade serous and endometrioid carcinomas were equivalent in terms of p53 mutations status, (2) if they shared similarities in other biomarkers and (3) if these biomarkers were similarly altered in recognizable early phases of the diseases (tubal intraepithelial carcinoma). In addition to p53, biomarkers traditionally upregulated in serous (WT-1 and p16<sup>ink4</sup>) and downregulated in endometrioid (PTEN and PAX2) carcinomas were studied.<sup>13,18-20</sup> The purpose of this strategy was to gain a greater understanding of high-grade Müllerian carcinomas in terms of (1) the need to separate them in the context of diagnostic pathology, (2) their potential origins and (3) the timing of these gene alterations, ie, their role in early (tubal intraepithelial carcinoma) and more widespread disease. In this study, we show these biomarkers are equivalent in their frequencies in all high-grade müllerian carcinomas. Further we will show that alterations in the

expression of these biomarkers characterize the earliest malignant phases of pelvic serous cancer.

## Materials and methods

### Case Selection and Analysis

The study was approved by the institutional review board at Brigham and Women's Hospital. We evaluated 89 consecutive cases of high-grade carcinoma retrieved from the pathology database between 2006 and 2008. In all, a protocol for extensively examining the fimbria and proximal tube was used (SEE-FIM protocol).<sup>8</sup> The histological diagnoses were verified by concurrent pathologic review by two coauthors (CPC and MRN) using a variation on published criteria.<sup>21</sup> Tumors with marked atypia, and papillary or prominent slit-forming gland-like spaces were designated as serous (Group 1). Tumors exhibiting sheet-like growth or focal endometrioid gland morphology with uniform nuclear features and lacking papillary, or slit-forming architecture were designated as high-grade endometrioid (Group 3). Tumors considered to have features intermediate between the two were designated as mixed serous and endometrioid pattern (Group 2). The presence or absence of a dominant ovarian mass, defined as greater than a twofold difference in the maximum recorded dimension between the two ovaries, and a tubal intraepithelial carcinoma were determined for each case, as described previously.<sup>13</sup> For statistical analysis, Groups 1 and 3 were compared using Fisher's exact test.

### Immunohistochemistry

Sections (5  $\mu$ m) were cut from formalin-fixed, paraffin-embedded blocks containing tumor tissue onto positively charged slides and baked overnight at 37°C. After sections were de-waxed and rehydrated, antigen retrieval was performed in a pressure cooker in 10 mM citrate buffer, pH 6.0. Immunostaining was performed with the following antibodies, respectively: mouse monoclonal anti-p53 antibody (clone DO-1; ImmunoTech, Westbrook, ME, USA); mouse anti-p16<sup>ink4</sup> antibody (clone 16P07; NeoMarkers, Fremont, CA, USA); and anti-WT-1 antibody (clone 6F-H2; DAKO, Carpinteria, CA, USA). The EnVision + System for use with mouse primary antibodies (DAKO) was subsequently used according to manufacturer's instructions and the slides were developed using 3, 3'-diaminobenzidine as a substrate and Gill's hematoxylin as a counterstain. For p53 and WT-1 immunohistochemistry, uniformly distributed and strong nuclear staining in at least 50% of the tumor cells was considered a positive, although diffuse positivity was the norm. Similarly, immunopositivity for p16<sup>ink4</sup> was scored positive when uniform and strong nuclear and cytoplasmic

staining was seen in virtually all of the tumor cells. If the staining was weak or patchy in distribution, it was classified as negative. Statistical comparison of the immunoprofiles of the high-grade serous and endometrioid carcinomas were performed using Fisher's exact test. PAX2 immunostaining was performed using an antibody from Invitrogen (Carlsbad, CA, USA; Cat# 71-6000) at 1:300. PTEN immunostaining was performed using the DAKO Monoclonal Mouse Anti-Human PTEN (Cat# M3627) at a dilution of 1:200. In both, cases were classified as negative if staining was seen in less than 5% of the cells in the tumor, heterogeneous if two discrete populations were negative and positive, and positive if over 95% of the tumor cells stained positive. This three step scoring strategy was used because the significance of staining distribution for these two biomarkers has not been clearly determined. In contrast, heterogeneous staining for p53, WT-1 and p16<sup>ink4</sup> was classified as negative on the basis of earlier experience.

### Analysis of p53 Mutations

Sections (5  $\mu$ m) were cut onto membrane slides (Leica Microsystems, Bannockburn, IL, USA). Tumor and control tissues were dissected manually using sterile scalpels or laser-capture microdissection (LCM). LCM was performed using the PALM microbeam instrument (P.A.L.M. Microlaser Technologies, Bernried, Germany). Genomic DNA was isolated, as described previously and amplified by polymerase chain reaction (PCR), using tailed primers designed to amplify p53 exons 1 through 11.<sup>22</sup> Secondary amplification was performed using T3 and T7 primers specific to the tail sequences used in the original amplification. T3 and T7 primers were subsequently used to sequence the PCR products. DNA sequences were analyzed using the Mutation Surveyor program (Soft Genetics, State College, PA, USA). To exclude spurious mutations introduced into somatic DNA as a consequence of formalin-fixation, all p53 mutation-positive exons were re-sequenced from a replicate amplified products.<sup>23</sup>

## Results

### Fimbrial Involvement and Dominant Ovarian Mass are Inversely Related in High-Grade Müllerian Carcinomas and are More Common in Serous and Endometrioid Carcinomas Respectively

Previously, we reported two parameters that correlated with assigning tumor origin for pelvic serous and endometrioid carcinomas, dominant ovarian mass and tubal intraepithelial carcinoma.<sup>13</sup> In this report, we subdivided the spectrum of tumors into three categories, including classic serous, one composed of tumors resembling serous and endometrioid and a third consisting of features characteristic of high-grade endometrioid tumors. The results are summarized in Table 1. Overall, 20 of 56, 5 of 20 of category 1 and 2 tumors were associated with a tubal intraepithelial carcinoma, compared with 1 of 13 category 3 (endometrioid) tumors, a difference (between categories 1 and 3) that bordered on significant ( $P=0.09$ ). In contrast, a significantly higher frequency of category 3 tumors were associated with a dominant ovarian mass ( $P=0.028$ ).

### High-Grade Müllerian Carcinomas have Equivalent Frequencies of (Positive) p53, WT-1, p16<sup>ink4</sup> and (Negative) PTEN and PAX2 Expression

Table 1 and Figures 1 and 2 summarize the immunostaining for the above biomarkers in the three groups of tumors. No significant differences in staining frequency were seen for any of the biomarkers including WT-1, which has been previously associated with serous carcinomas. Staining frequencies for p53 (85–93%, not shown in table), p16<sup>ink4</sup> (69–75%), WT-1 (70–98%) and PAX2 (negative; 67–75%) were high in all categories, with  $P$ -values ranging from 0.58 to 1.0. Similarly, the rate of negative staining for PTEN (5–12%) was comparable and did not distinguish high-grade endometrioid carcinomas from the other two categories. However, when the numbers demonstrating either negative or focally negative (heterogeneous) were combined, the difference between groups 1 and 3

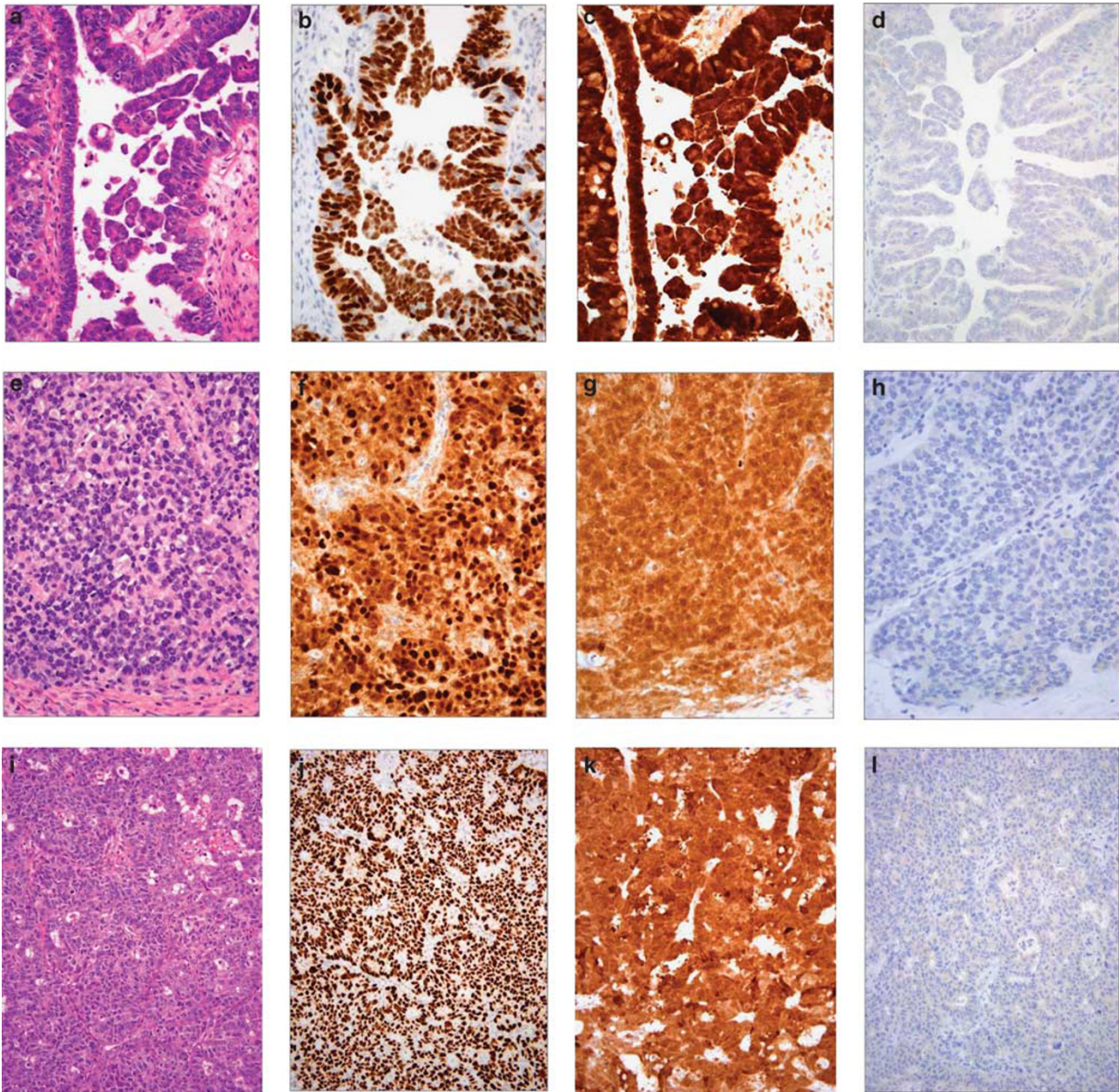
**Table 1** Summary data from immunohistochemical analysis of high-grade serous (1), mixed serous and endometrioid (2) and endometrioid (3) carcinomas

Tumor type	No.	TIC (%)	DOM (%)	P53 Mut (%)	p16 <sup>ink4</sup> (%)	WT-1 (%)	PTEN <sup>a</sup>			Pax-2 <sup>a</sup>		
							Neg	Het	Pos	Neg	Het	Pos
1	56	20 (36)	19 (34)	51 (93)	40 (71)	55 (98)	6	28	22	37	6	9
2	20	5 (25)	9 (45)	15 (75)	15 (75)	14 (70)	3	9	7	13	2	3
3	13	1 (8)	9 (69)	12 (92)	9 (69)	12 (92)	5	6	1	8	2	2
P-value (1 vs 3 <sup>b</sup> )		0.09	0.028	1.0	1.0	0.25	0.048			1.0		

TIC, tubal intraepithelial carcinoma; DOM, dominant ovarian mass (see text); Neg, less than 5% of tumor cells stained positive; Pos, greater than 95% of tumor cells stained positive; Het, tumor contained discrete positive and negative staining cell populations.

<sup>a</sup>Negative and heterogeneous staining were combined for statistical analysis.

<sup>b</sup>Analyzed by Fisher exact test.



**Figure 1** Immunohistochemical analysis of high-grade serous and endometrioid carcinomas. Hematoxylin and eosin-stained sections of high-grade serous, mixed serous and endometrioid and endometrioid carcinomas (a, e, i) along with immunohistochemical stains for p53 (b, f, j), p16<sup>ink4</sup> (c, g, k) and PAX2 (d, h, l) are illustrated. The staining pattern for PAX2 in normal fallopian tube is shown in Figure 3b.

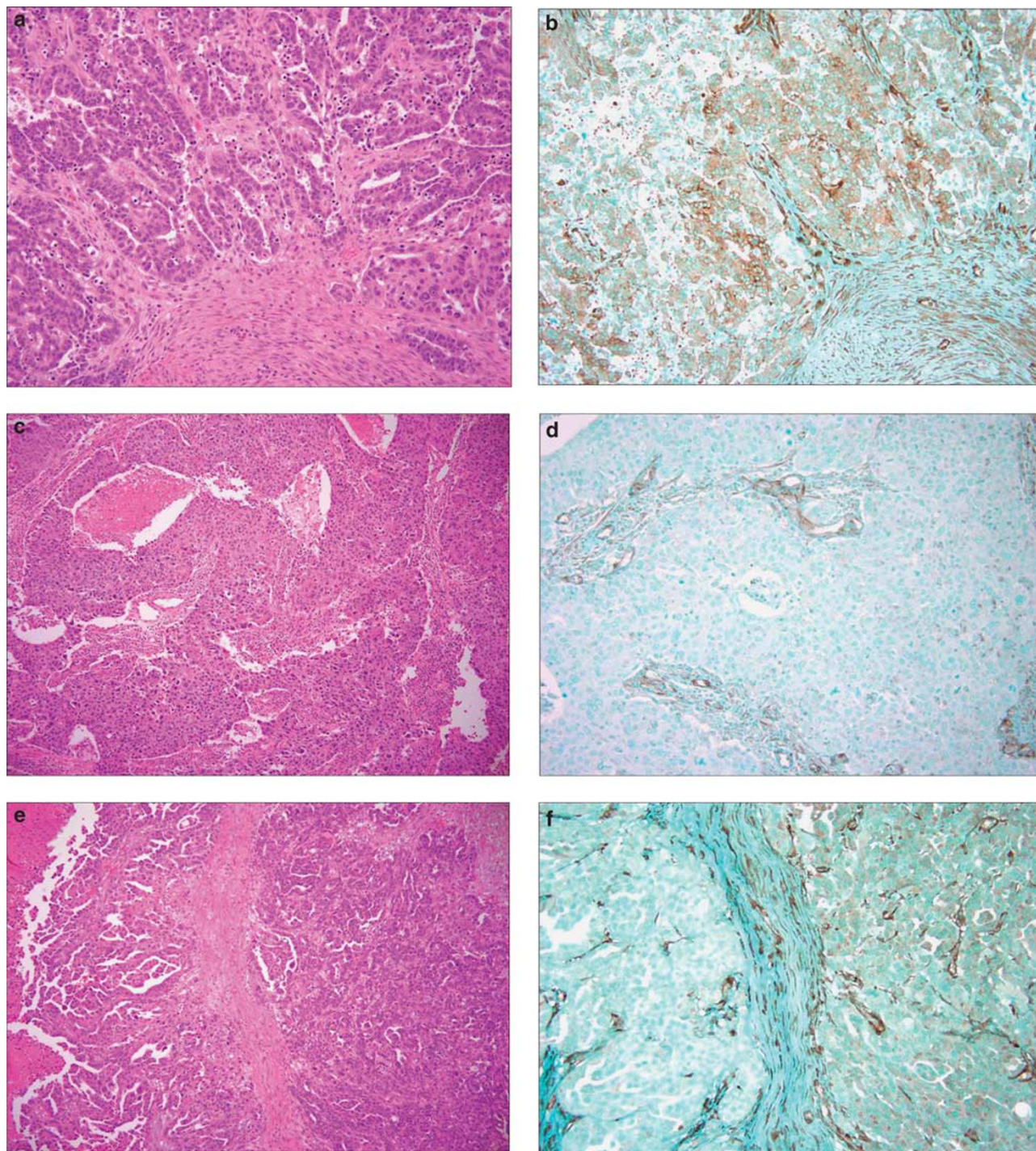
were significant at  $P=0.048$  (Table 1), suggesting a higher frequency of heterogeneous or negative staining in high-grade endometrioid carcinomas. However, high frequencies of heterogeneous PTEN staining were also seen in Groups 1 and 2. In contrast, staining pattern frequencies for PAX2 staining were equivalent in all groups ( $P=1.0$ ).

#### Frequency of *p53* Mutations is Similar in High-Grade Serous and Endometrioid Carcinomas

As a high proportion of high-grade serous and endometrioid carcinomas were immunopositive for

*p53*, we next sought to determine the frequency of *p53* mutations (Tables 1 and 2). We detected *p53* mutations in 89% of the entire group including 93, 75 and 92% of groups 1, 2 and 3.

The majority of the *p53* mutations were missense mutations (Table 2). Missense mutations were detected in from 67 to 83% of tumors and were not significantly associated with any one group. The presence of missense mutations correlated highly with *p53* overexpression on immunohistochemistry as 85% of these mutations were associated with strong staining. The remaining mutations were nonsense, insertion, deletion and splice donor/



**Figure 2** Paired hematoxylin and eosin (a, c, e) and PTEN immunostains (b, d, f) of high-grade Müllerian carcinomas, demonstrating diffusely positive (b), negative (d) and heterogeneous (f) staining. The latter typically designates discrete units of epithelium with juxtaposed positive (right) and negative (left) staining (f). The staining pattern for PTEN in normal fallopian tube is shown in Figure 3c.

**Table 2** Types of p53 mutations associated with different high-grade tumor groups

Tumor type	P53 mutation	Missense (%)	Nonsense	Splice	Insertion	Deletion
1	51/56	34 (67)	1	5	3	8
2	15/20	10 (67)	1	2	0	2
3	12/13	10 (83)	0	0	0	2

1, serous carcinoma; 2, mixed serous and endometrioid features; 3, endometrioid carcinoma.

acceptor-site mutations. The majority of the *p53* mutations were present in exons 5–8, which encode for the DNA-binding domain of *p53*, accounting for 83% of all *p53* mutations detected.

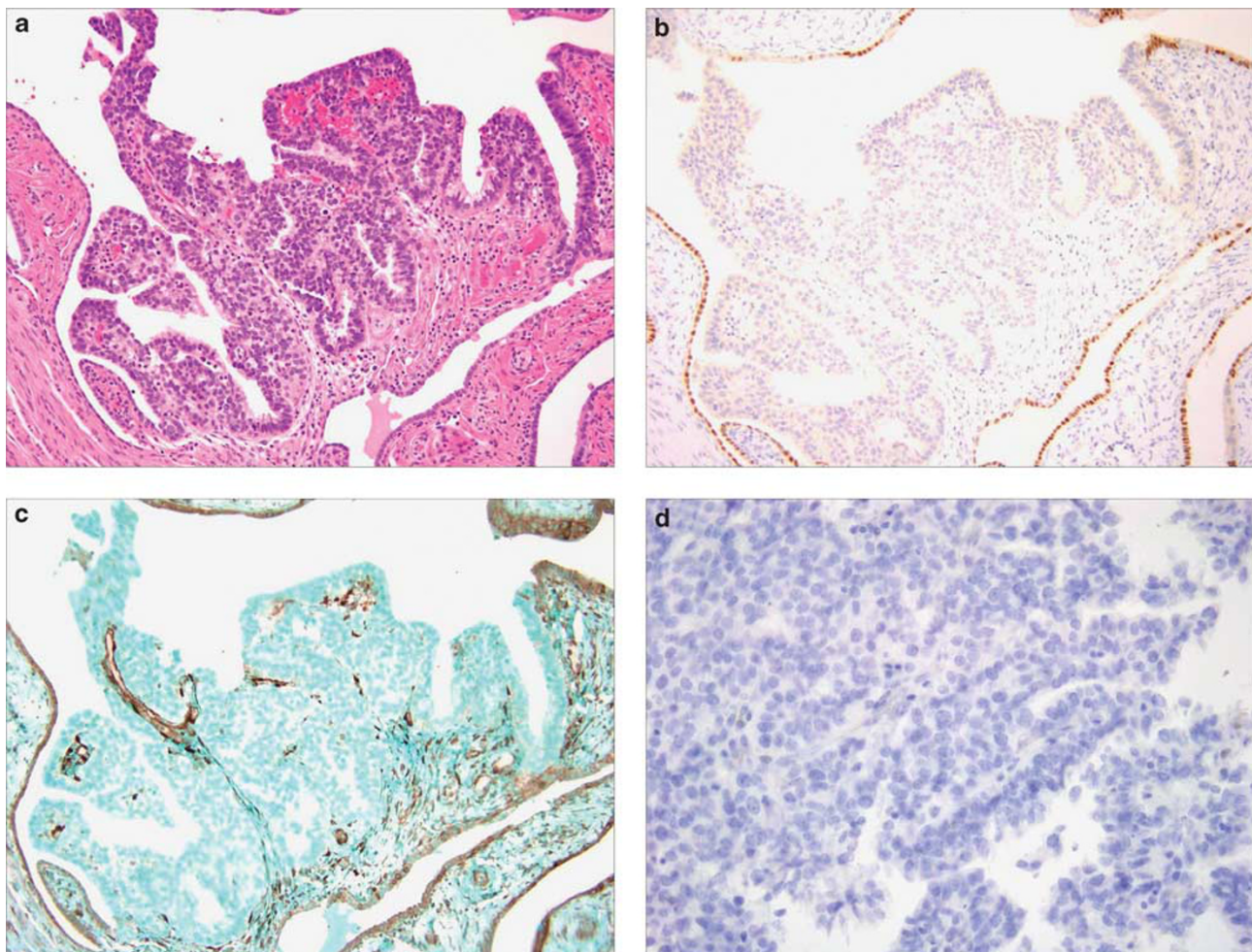
#### Alterations in Expression of *p53*, *Pax2* and *PTEN* Characterized Earlier forms of Serous Carcinoma (Tubal Intraepithelial Carcinoma)

Analysis of serous tubal intraepithelial carcinomas for the above biomarkers was performed to determine if they shared the same immunophenotype as the concomitant advanced malignancies (Figure 3). Fifteen blocks from 12 cases were analyzed. Eleven cases demonstrated strong staining for *p53*. Seven cases contained invasive carcinoma elsewhere in the same section as the intraepithelial carcinoma. In all 12 cases, *PAX2* staining was negative in the intraepithelial carcinoma, although in two cases heterogeneous staining was seen in another block. In four blocks from four cases, *PTEN* staining was absent (negative). In four other cases, *PTEN* staining of tubal intraepithelial carcinoma was heteroge-

neous. In 9 of 10 cases, the coexisting invasive carcinomas were *PAX2* negative; in another, *PAX2* staining was heterogeneous. The correlation between *PTEN* staining in the intraepithelial and invasive carcinomas varied, with some tumors losing *PTEN* expression, whereas others demonstrated heterogeneous staining (Table 3, Figure 3).

#### Discussion

This study has confirmed earlier observations that the origin of high-grade Müllerian carcinomas is diverse, including the ovary, distal fallopian tube and possibly, other sites. The serous or mixed serous and endometrioid carcinoma phenotype correlates with the presence of a tubal intraepithelial carcinoma with involvement of both ovaries in an equal distribution, whereas high-grade endometrioid carcinoma tumors are uncommonly associated with tubal intraepithelial carcinoma and predominate in one ovary over the other (Table 1). However, as shown in Table 1, there is considerable 'leakage' of these two variables across all three histological



**Figure 3** Absence of *PAX2* and *PTEN* staining in tubal intraepithelial carcinoma. (a) Hematoxylin and eosin staining, (b) *PAX2* and (c) *PTEN* staining. (d) Staining for *p53* is completely negative, consistent with a deletion mutation of the *p53* antigenic target.

**Table 3** Pax-2 and PTEN staining of tubal intraepithelial carcinoma

Case	P53	Pax-2	PTEN	Invasive cancer (same section)
1	Pos	Neg	Het	PTEN Pos/Pax-2 Neg
2	Pos	Neg	Neg	None
3 block 1	Pos	Neg	Pos	PTEN Het/Pax-2 Neg
3 block 2	Pos	Neg	Pos	PTEN Het/Pax-2 Neg
3 block 3	Pos	Het	Pos	PTEN Pos/Pax-2 Neg
4	Pos	Neg	Neg	PTEN Het/Pax-2 Neg
5	Pos	Neg	Het	PTEN Het/Pax-2 Neg
6	Neg	Neg	Neg	None
7	Pos	Neg	Pos	None
8	Pos	Neg	Pos	None
9	Pos	Neg	Het	None
10 block 1	Pos	Het	Het	PTEN Neg/Pax-2 Het
10 block 2	Pos	Neg	Pos	PTEN Het/Pax-2 Neg
11	Pos	Neg	Neg	PTEN Neg/Pax-2 Neg
12	Pos	Neg	Pos	PTEN Het/Pax-2 Neg

Het, heterogeneous staining; Pos, positive; Neg, negative.

groups. Thus, a diagnostic pathologist cannot expect histological criteria to segregate high-grade müllerian carcinomas to specific organs (tube *vs* ovary). Nevertheless, the biomarker expression patterns, seen in these tumors and in early tubal malignancies, combined with the coexisting conditions such as tubal intraepithelial carcinoma and dominant ovarian mass, raise new and compelling questions about the classification and pathogenesis of high-grade müllerian carcinomas. The immunohistochemical studies draw high-grade endometrioid and serous carcinomas closer together by shared disturbances in gene expression. However, there is reason to assume that shared pathogenetic mechanisms operate in both the ovary and tube and possibly, other sites.

As illustrated in Table 1, none of the three tumor groups, including those with classic features of high-grade endometrioid carcinoma, could be distinguished on the basis of the expression of three biomarkers that have been recently studied in ovarian cancer. p53, WT-1 and p16<sup>ink4</sup>, which are generally heterogeneous in low-grade endometrioid and typically diffusely positive in serous carcinomas, were strongly positive in a high frequency of each tumor category, an observation that has been appreciated previously.<sup>13,15,17,19</sup> Nearly 90% of all tumors exhibited mutations in p53, a gene typically assigned to the high-grade serous carcinomas. Recent studies have identified a subset of high-grade endometrioid carcinomas that contain p53 mutations, separating this group from other endometrioid carcinomas.<sup>19</sup> Although p53 mutation-positive tumors were classified initially as an uncommon subset of ovarian endometrioid carcinomas, the relative frequency of this group may be a function of differences in tumor classification.<sup>19</sup> Most of the high-grade endometrioid carcinomas in this study contained mutations in p53.

Of considerable interest were the expression patterns of PAX2 and PTEN, both in intraepithelial and invasive carcinomas. PAX2 is a relatively new biomarker to be studied in ovarian cancer and its relationship to these tumors is evolving. PAX2 has been most intensively studied in the kidney. It is designated a homeogene that is critical to renal development and is expressed in approximately 60% of renal cell carcinomas.<sup>22</sup> Its role in müllerian neoplasms is less clear. PAX2 is strongly expressed in tubal secretory cells and in similar cells in ovarian cortical inclusion cysts. Some reports have described PAX2 staining in ovarian serous carcinomas; however, more recent studies have noted a stronger likelihood of staining in low-grade *versus* high-grade serous carcinomas.<sup>20,24</sup> Our interest in this gene stemmed from a recent report by one of us (GLM) that expression of both PAX2 and PTEN are absent in the early phases of endometrial carcinogenesis, including endometrial intraepithelial neoplasia.<sup>25</sup> On the basis of this information, we examined the expression of both in the tumors reported in this study.

We used a three stage system for both PAX2 and PTEN scoring because we had relatively little experience with these antibodies but have observed staining in both to be heterogeneous. PAX2 expression was either completely lost or stained less than 5% of tumor cells in over 70% of tumors. In contrast, diffuse expression of this biomarker was uncommon, seen in only 17% of all cases. The remaining 12% exhibited various indices of staining, more in keeping with variable regulation of gene expression. Expression of PTEN also varied but less so. Only 17% scored as completely negative, but only 34% were diffusely positive and nearly one-half exhibited heterogeneous staining. Thus, the varied staining patterns of both PTEN and PAX2 suggest more than one mechanism may be operative, including loss of function by the loss of all or part of the encoding gene, and differential regulation of gene expression, either genetic or epigenetic, as observed previously (Mutter GL, unpublished). This is in contrast to the common observation that the loss of p53 function is attributed to both mutation and loss of heterozygosity.<sup>26</sup> The biological significance of either loss or partial loss of PAX2 and PTEN staining in invasive or advanced carcinomas remains uncertain, in the context of the extensive and widespread disturbances in gene expression that characterizes these tumors.<sup>27</sup>

A compelling finding in this study was the nearly consistent loss or marked reduction in PAX2 staining in a high percentage of not only advanced malignancies but tubal intraepithelial carcinomas (Table 3, Figure 3). In the face of the overall high frequency of PAX2 loss shown in Table 1, this is not unexpected. Moreover, a causal relationship between tubal intraepithelial carcinomas and concurrent remote tumors has been previously established by the sharing of identical mutations in p53.<sup>11,12</sup>

However, notwithstanding the fact that intraepithelial carcinomas are considered to have malignant potential (proven further by the advanced tumors with which they associate) staining of PAX2 and PTEN support the concept that the reduction in expression of these genes occurs in the earlier time points of the malignant process. Moreover, it links PAX2—a gene whose perturbations are associated with endometrioid carcinoma—to serous carcinogenesis.<sup>26</sup>

The frequency of PTEN-negative staining in intraepithelial carcinomas was lower (33%, Table 3, Figure 3); however, it was heterogeneous in four additional cases and the adjacent invasive carcinomas were often heterogeneous in their PTEN staining (Table 1, Figure 2). Thus, there is evidence to suggest that alterations in the expression of both PTEN and PAX2 could be involved in the early stages of serous carcinogenesis, similar to endometrioid carcinomas of the uterus.<sup>26</sup> In this context, the immunostaining results raise two other important questions. The first is whether PTEN and PAX2 are lost before malignancy, as seen in the endometrial model, or are coincident with the emergence of malignancy. We have recently identified discrete benign appearing secretory cell outgrowths in the fallopian tube, many of which are associated with loss of PAX2 (and sometime PTEN) expression, presenting a compelling corollary to the observations in the endometrium.<sup>28</sup> Thus, there is the real possibility that some of the genes downregulated in serous cancer become altered before the onset of the malignant phenotype. The second is whether a similar pathway that includes downregulation of these genes can be elucidated in a site other than the fallopian tube. Efforts to identify p53-positive ovarian cortical inclusion cysts have largely been unsuccessful. However, an alternative model of ovarian serous carcinogenesis could include the initial downregulation of PTEN, PAX2 or other genes in endometriosis, ovarian surface epithelium or cortical müllerian inclusions, setting the stage for malignancy with subsequent loss of p53 function. A variation of this theme has been proposed for ovarian surface epithelium, although based on limited cases.<sup>29</sup> Whether such a scenario exists awaits a more exhaustive study of the benign ovarian cortex in women at risk for ovarian cancer and the general population. If the above studies are fruitful and confirm this report, PTEN and PAX2 dysregulation may join p53 as important early events in the genesis of the most common and lethal ovarian cancers in women.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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