Original Articles

Fractional allelic loss as a potential biomarker of risk prediction in early-stage mucinous ovarian tumors of low malignant potential

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Summary

Ovarian tumors of low malignant potential (LMP) appear to be intermediate between adenomas and ovarian carcinomas. Such tumors are often associated with a significantly better prognosis than for ovarian carcinomas. However, a subset of LMPs can progress and become lethal even in patients with early-stage disease. In order to seek sensitive diagnostic tools to monitor patients after surgical therapy, we performed a genome-wide scan for LOH in 37 early-stage mucinous LMPs using 91 polymorphic microsatellite markers at an average interval of 50 cM across all of the human chromosomes and 25 LOH markers reported to be associated with ovarian carcinoma. Fractional allelic loss (FAL) values were calculated as (loci scored with LOH)/(total informative loci) for each sample. With respect to tumor recurrence, high FAL values were more frequent in recurrent tumors than in non-recurrent tumors. Using the screening markers, FAL values for recurrent tumors were significantly higher than for non-recurrent tumors (19.8% vs 6.3%, respectively, $p < 0.0001$). Similar results were obtained using the hotspot markers (22.2% vs 7.1%, respectively, $p < 0.0001$). A significant correlation between FAL values obtained using screening markers and those based on hotspot markers was observed ($R = 0.460$, $p = 0.003$). Our findings suggest that a specific type of genetic instability (i.e., chromosomal instability, CIN) may exist in mucinous LMPs, and that this instability may indicate tumors with an aggressive biological nature. Therefore, FAL values may represent a new biomarker for risk prediction in early-stage mucinous LMP tumors.

Key words: Ovarian Carcinoma; Mucinous LMP; Loss of heterozygosity (LOH); Fractional allelic loss.

Introduction

Among gynecologic malignancies, ovarian carcinoma is the leading cause of death [1]. Ovarian tumors of low malignant potential (LMP), also referred to as borderline ovarian tumors, are a distinct subtype of ovarian epithelial tumors and are thought to represent an intermediate stage between clearly benign tumors and invasive carcinomas. While LMPs are characterized by the absence of ovarian stromal invasion, they retain the ability to metastasize. The incidence of LMP ranges from 9% to 16.3% of epithelial neoplasms, and they occur primarily in Stage I disease [2]. Serous and mucinous tumors comprise the vast majority of cases, although endometrioid, clear cell, and Brenner-type tumors have also been described [3]. While most patients with LMPs can be cured by surgical excision, approximately 15% experience tumor recurrence and die from their disease [4, 5]. Although tumor recurrence has been associated with advanced-stage disease [4, 6], many patients with advanced lesions suffer no tumor relapse while other patients with early-stage LMPs develop tumor recurrence. Thus, the identification of patients most likely to suffer recurrence after primary surgical therapy remains an important problem.

The poor outcome for some LMP patients is largely due to the lack of sensitive diagnostic tools for the monitoring of patients after surgical treatment, with clinical and pathological parameters, often difficult to assess, during therapeutic decision making. In the search for more reliable prognostic indicators, a few investigators have focused on biological markers that might be predictive of LMP development [7-9].

As little is known about genetic alterations associated with LMPs, it has remained unclear which molecular markers influenced the fate of these patients. Among the various types of genetic alterations involved in ovarian carcinoma development and progression, loss of heterozygosity (LOH) of particular chromosomal regions is thought to indicate the deletion of normally resident tumor suppressor genes [10, 11]. Thus, it is possible that the loss of specific alleles may act as a diagnostic marker of prognosis. Recently, we reported that fractional allelic loss (FAL) values could predict poor outcome in ovarian carcinoma patients [12]. To determine whether FAL values could be used to predict recurrence in early-stage mucinous LMP patients, we performed a genome-wide scan of LOH in 37 Stage I mucinous LMPs using 91 polymorphic microsatellite markers at an average interval of 50 cM throughout all human chromosomes (screening markers) as well as 25 LOH markers reported to be associated with ovarian carcinoma (hotspot markers).

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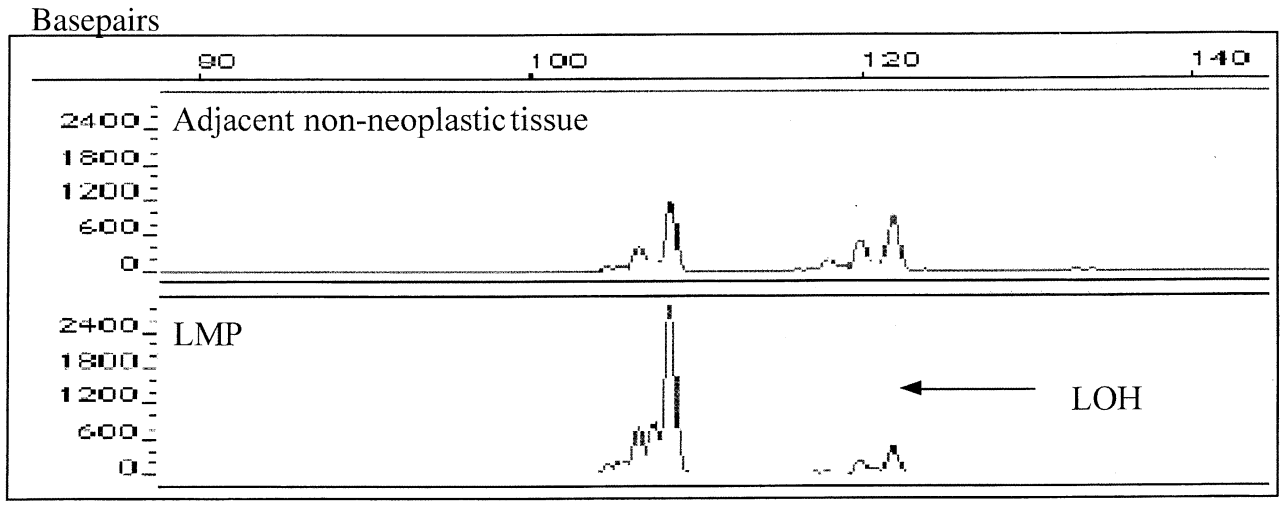

Figure 1. — Representative example of loss of heterozygosity (LOH).

Material and Methods

Patients and tumor samples

Thirty-seven mucinous LMP tissues and adjacent non-neoplastic tissues were obtained from archival pathological specimens at Shimane University Hospital and the Institute of Japan Surgical Pathology. Written informed consent for this study was obtained for each case individually. Acquisition of tissue specimens and clinical information was approved by an institutional review board (Shimane University). Diagnostic verification, tumor subtyping, and grading were performed independently by three certified pathologists. LMPs were diagnosed on the basis of conventional histopathologic criteria [13], and graded according to grading criteria recommended by the International Federation of Gynecology and Obstetrics.

All tumor samples used in this study were of mucinous subtype. The incidence of mucinous LMP in Japan is high compared to Western countries [14, 15]. All patients had Stage I tumors. Unilateral salpingo-oophorectomy was performed on patients who presented with Stage I disease and none of the patients received adjuvant chemotherapy. Follow-ups for all patients included in the survival analysis were updated on January 25, 2003 (median follow-up time 103 months; range, 60-144 months). At that time, five patients had progressed to well-differentiated ovarian carcinoma and 32 had not progressed.

DNA extraction

Paraffin-embedded tissues were sectioned at a thickness of 5 mm and stained with hematoxylin and eosin. Cancerous and non-neoplastic portions were collected separately using a 29-gauge needle and an MK1 micromanipulator (Singer Instruments, Roadwater, UK) under a dissecting microscope. Dissected tissues were collected in Eppendorf tubes and incubated overnight at 58°C in digestion mixture (0.01 M NaCl; 0.5 M Tris-HCl, pH 8.0; 20 mM EDTA; 0.05% Tween20R; 0.1 mg/ml proteinase K). Samples were then heated to 95°C for 10 min to inactivate proteinase K activity, after which DNA was extracted by phenol/chloroform treatment and ethanol precipitation.

Microsatellite markers

We examined LMP samples for the presence of LOH using dye-labeled microsatellite markers, including 25 markers for which LOH is reported to be frequent in ovarian carcinoma (hotspot markers). Primer information for the hotspot markers has been described previously [16]. Further LOH analysis of the LMP samples was carried out using 91 markers located an average of 50 cM apart and distributed throughout the human genome (screening markers) (CHLC/Weber Human Screening Set 6A, Research Genetics, Huntsville, AL).

PCR amplification and analysis of loss of heterozygosity

PCR reactions were performed in total volumes of 10 µl containing 25-50 ng DNA, dNTPs at a final concentration of 20 µM, 0.4 µM each primer, and 0.25 U *Ex-Taq* DNA polymerase (Takara Shuzo, Shiga, Japan) or Platinum *Taq* DNA polymerase (GIBCO BRL, Rockville, MD). After heating for 10 min at 94°C, PCR was performed for 45 cycles of 1 min each at 94°C, at the appropriate annealing temperature, and at 72°C, followed by 72°C for 10 min. After denaturation at 94°C for 2 min, PCR products were subjected to electrophoresis using Performance Optimized Polymer 4 in a 310 Genetic Analyzer (Applied Biosystems, Foster, CA). LOH analysis was performed by Gene Scan version 2.1. LOH was quantitatively assessed by calculating the LOH index, which was defined as the allele ratio in the normal tissue sample divided by the allele ratio in the tumor tissue sample. The allele ratio was calculated as the peak height of the smaller allele divided by the peak height of the larger allele. If the LOH index was less than 0.5 or more than 2.0, the case was considered to show LOH. A representative example of LOH is shown in Figure 1.

Statistical analysis

Data analysis was performed using the Statview version 5 statistical software package. Continuous variables were analyzed using Student's t-test. The correlation coefficient (r) between different parameters was determined by simple regression analysis; all values are two-sided and p values less than 0.05 are considered statistically significant.

Results

FAL values using screening markers

Fractional allelic loss (FAL) values were calculated for each sample as (loci scored with LOH)/(total informative loci). FAL values from screening markers

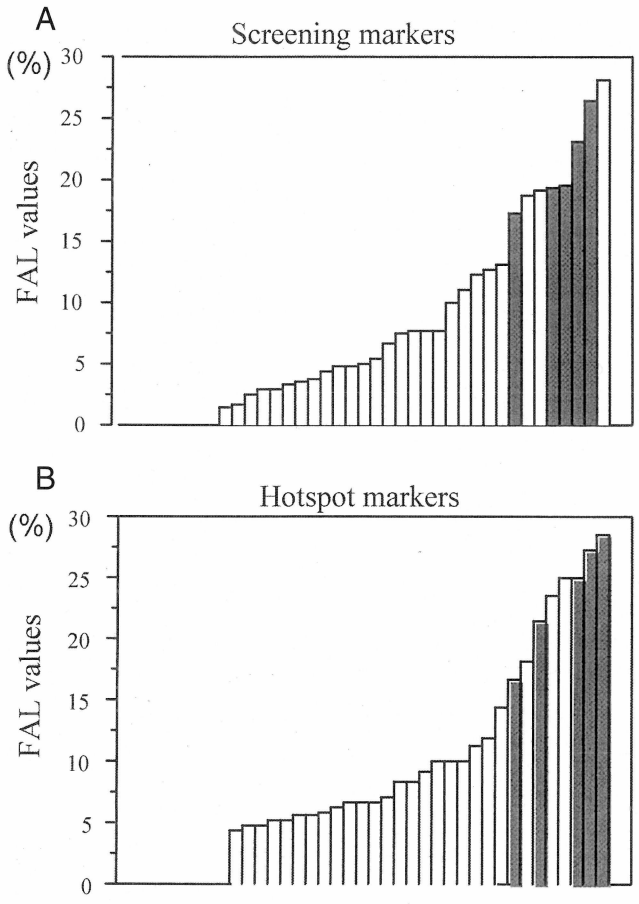

Figure 2. — FAL values arranged in the order of magnitude for screening markers (A) and hotspot markers (B). Black and white bars are recurrent and non-recurrent tumors, respectively.

arranged in order of magnitude for the 37 mucinous LMPs were plotted in a graph (Figure 2A). Results showed that for the screening markers, 32 out of 37 tumors (86.4%) demonstrated at least one LOH event. FAL values for all mucinous LMP samples ranged from 0 to 28.2% with a mean of 8.5%. With respect to tumor recurrence, FAL values were higher in tumors that exhibited recurrence than in those that did not show recurrence. FAL values ranged from 12.8 to 26.5% with a mean of 19.8%, in recurrent tumors, compared to 0 to 28.2% with a mean of 6.3%, in non-recurrent tumors ($p < 0.0001$) (Table 1).

Table 1. — *FAL (Fractional allelic loss) values (±SD) and recurrence status.*

	FAL values (%)	
	Screening markers	Hotspot markers
Recurrence	19.8 (± 4.7) (n = 5)[1]	22.2 (± 5.8) (n = 5)[2]
Non-Recurrence	6.3 (± 6.5) (n = 32)	7.1 (± 6.2) (n = 32)

[1, 2] FAL values were significantly higher in tumors that exhibited recurrence than in those that did not show recurrence ($p < 0.0001$).

FAL values using hotspot markers

FAL values from hotspot markers arranged in order of magnitude for the 37 mucinous LMPs were plotted in a graph (Figure 2B). For the hotspot markers, 35 out of 37 tumors (94.6%) demonstrated at least one LOH event. For all mucinous LMPs, FAL values ranged from 0 to 28.6% with a mean of 9.6%. With respect to tumor recurrence, FAL values based on hotspot markers were also higher in recurrent than non-recurrent tumors. FAL values ranged from 14.3 to 28.6% with a mean of 22.2%, in recurrent tumors, and from 0 to 25.3% with a mean of 7.1%, in non-recurrent tumors ($p < 0.0001$) (Table 1).

Relationship between FAL values in screening markers and that in hotspot markers

A significant correlation between FAL values obtained using screening marker $p = 0.003$) (Figure 3). These findings suggested that a specific type of genetic instability (i.e., CIN) was associated with LMPs, and that increased instability is related to a more aggressive tumor phenotype.

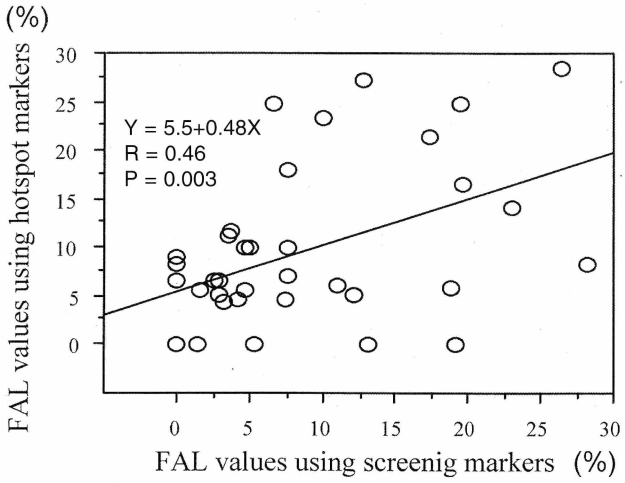

Figure 3. — A significant correlation was observed between FAL values obtained using screening markers and those obtained using hotspot markers ($R = 0.460$, $p = 0.003$).

Discussion

Although early-stage LMPs are often associated with a significantly better prognosis than advanced-stage disease [4, 6], a subset of early-stage LMPs can progress and become lethal. The ability to predict the most likely postoperative course has become increasingly important for LMP patients as high-risk patients would benefit from adjuvant chemotherapy. At present, routine postoperative decisions for LMP patients rely solely on the consideration of conventional prognostic factors, such as clinical stage. In the current study, we assessed FAL values using microsatellite analysis and found that a high FAL value could predict the risk of postoperative progression for early-stage mucinous LMP patients. We first, found that high FAL values based on genome-wide screening markers were significantly associated with tumor recur-

rence. Recent studies of colon cancer cells reported that these cells exhibited a specific type of genetic instability (i.e., CIN). This indicated that the CIN phenotype may be a causative factor in tumor development, and may reflect from defects in proteins participating in mitotic checkpoint control [17]. The striking association between tumors with a high rate of LOH and poor survival, as observed in our study, may be explained by the possibility that high FAL values reflect an aggressive tumor phenotype. Our findings support the hypothesis that tumor-specific types of genetic instability exist, and that this instability has a role in driving tumor development [18]. FAL values were also assessed using 25 sets of microsatellite markers that are known to be tightly linked to tumor suppressor genes or are reported to be associated with ovarian carcinoma (hotspot markers). Our results showed the FAL values based on hotspot markers also predicted tumor recurrence, such that high FAL values based on hotspot markers were also significantly associated with tumor recurrence. Interestingly, FAL values obtained using screening markers or hotspot markers were significantly correlated (Figure 3). Due to considerations of cost and speed, it would clearly be of benefit to use only a small number of microsatellite markers to identify FAL values in the clinical setting. Our results suggested that it should be possible to select a relatively small but effective set of microsatellite markers. Our results also suggested that high FAL values were associated not only with tumor progression, but also with an aggressive clinical phenotype. Since ovarian carcinomas have a high rate of LOH events compared to LMPs in our previous report [16], LMP tumors with high FAL values may be precursors of ovarian carcinomas with the resultant poor clinical outcome.

Our study had several limitations, including the relatively small number of patients studied due to the logistics of long-term patient follow-up. Another limitation was that LMPs with microinvasion were not included in our study. Larger studies with more cases, including LMPs with microinvasion, as well as selecting a smaller set of effective microsatellite markers, are needed to clarify the significance of FAL values in the clinical setting. Because histopathological examination alone cannot select between mucinous LMPs that will relapse from early-stage mucinous LMPs [19], FAL values using LOH analysis may be a useful tool to select such tumors. It is also clear that we need to be vigilant when following-up mucinous LMPs patients with high FAL values after surgery, even if those patients have only Stage I disease.

In conclusion, the present study described for the first time the use of microsatellite analysis to predict recurrence for early-stage mucinous LMP patients. FAL values represent a potential new biomarker for risk prediction in early-stage mucinous LMPs.

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