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Localization of a novel susceptibility gene for familial ovarian cancer to chromosome 3p22–p25

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We performed genome-wide linkage analysis in 58 patients and nine unaffected members among 28 families with no mutation in *BRCA1* or *BRCA2*, employing a set of 410 microsatellite markers. We initially screened the whole genome, including the X chromosome, by a non-parametric method using the GENEHUNTER program. As a result, chromosome 3p22–p25 showed a suggestive score for linkage [LOD = 3.49 and non-parametric LOD (NPL) = 2.77 at *D3S3611*] based on a multipoint analysis. Additionally, based on a two-point analysis using dense markers, this 3p22–p25 region showed a *P*-value < 0.05 at 10 markers and there is suggestive evidence for linkage at two markers within ~19 cM (NPL = 2.60 and 2.49 at *D3S1597* and *D3S3611*, respectively). To explore whether the candidate gene in this 3p22–p25 region contributed to carcinogenesis of familial ovarian cancer in a similar fashion to the tumor suppressor gene, we performed loss of heterozygosity (LOH) analysis. It was observed that the frequency of LOH at four markers in this region was >50% only in tumor tissues from patients with no mutation in *BRCA1* or *BRCA2*, not in those with a *BRCA1* mutation.

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INTRODUCTION

Ovarian cancer is the most lethal among gynecological malignant cancers. Approximately 5–10% of cases are thought to have a hereditary basis (1), and a positive family history of ovarian cancer is one of the strongest and most consistent of the risk factors for development of the disease. It has been reported that first-degree relatives of ovarian cancer patients were found to be at a 2- to 4-fold increased risk of developing the disease (2,3). Familial ovarian cancer occurs in two distinct groups; site-specific ovarian cancer families and breast-ovarian cancer families (4).

Until now, the *BRCA1* gene on chromosome 17q21 and the *BRCA2* gene on chromosome 13q12–q13 have been identified by positional cloning methods followed by genetic linkage analysis for familial breast cancer (5,6). Germline mutations of *BRCA1* are predicted to be responsible for ~45% of breast cancer families and 80% of breast-ovarian cancer families (7–9). Both male and female *BRCA2* carriers have a high risk of early-onset breast cancer; however, ovarian cancer was initially thought to be a much less prominent feature of these families, but it is now thought that *BRCA2* may account for as much as 10–35% of familial ovarian cancers (9,10). Recently, it has been reported that about half of ovarian cancer families are not caused by these two genes (11,12). These data suggest that the contribution of other ovarian cancer susceptibility genes cannot be excluded. In a previous study, we reported 11 independent *BRCA1* mutations in 26 patients of 12 families: nine patients in four site-specific ovarian cancer families and 17 patients in eight breast-ovarian cancer families. There were no significant differences of average age at diagnosis between *BRCA1* cases and sporadic cases, and 24 of 25 patients with germline mutation of *BRCA1* had a serous type of adenocarcinoma (13,14).

In sporadic ovarian cancer, several interesting tumor suppressor genes, such as *NOEY2*, *PTEN* and *OVCA1, 2*, have been identified (15–18). However, little evidence has been reported suggesting that these genes are important in the pathogenesis of sporadic ovarian cancers, and their roles in the development of familial ovarian cancer are still unknown.

A segregation analysis suggested that familial ovarian cancer is due to low penetrant, dominant or recessive genes (19–21). The observation that, in general, ovarian cancer clusters are smaller than those observed in other cancers (e.g. breast and colon cancers) could be explained either by a lower recall of family history among females or by the model that ovarian cancer predisposition is due to a lower-penetrance gene than other cancer susceptibility genes. Eccles *et al.* (19) estimated a lower penetrance (50%) of these susceptibility genes under the best fitting dominant model.

In this present study, we analyzed genetic alterations of *BRCA1* and *BRCA2* in familial ovarian cancer patients. In addition, we performed genome-wide linkage analysis in families in which no mutation was found in *BRCA1* or *BRCA2* to identify novel susceptibility genes of familial ovarian cancer other than *BRCA1* and *BRCA2*.

RESULTS

Patients

We ascertained and performed direct sequencing of available patients with 196 epithelial familial ovarian cancer patients in all 81 families for mutational analysis in *BRCA1* and *BRCA2*. Among the 81 ovarian cancer families, we found 39 and five families carrying germline mutations of *BRCA1* and *BRCA2*, respectively. In 24 independent mutations of *BRCA1*, 18 mutations of *BRCA1* had never been described previously (M. Sekine, H. Nagata, S. Tsuji, Y. Hirai, S. Fujimoto, M. Hatae, I. Kobayashi, T. Fujii, I. Nagata, K. Ushijima, K. Obata, M. Suzuki, M. Yoshinaga, N. Umesaki, S. Satoh, T. Enomoto, S. Motoyama, K. Tanaka and The Japanese Familial Ovarian Cancer Study Group, manuscript in preparation). No germline mutation of *BRCA1* or *BRCA2* was detected in 78 affected patients in 37 families. Regarding the other cancers in these 37 families, family history was analyzed in the third degree relatives and second degree relatives for breast cancer and other histologic types of all cancers, respectively. Five families had one breast cancer patient other than ovarian cancer patients. The mean age at diagnosis of these five breast cancer patients was 48.0 years. In addition, two pairs of individual cancers, hepatic and gallbladder cancer, and stomach and hepatic cancer, were found in two independent families, and one histologic type of cancer, e.g. stomach, esophageal, hepatic, oral cavity, pancreatic, rectal, lung or uterine cancer, was observed in 12 independent families. Four ovarian cancer patients had a personal history of other types of previously diagnosed cancers, such as breast, bladder, endometrial and pancreatic cancer.

Table 1 demonstrates the clinical characteristics of 78 patients with no mutation in *BRCA1* or *BRCA2*, and 1299 control patients from the cancer registry of Niigata in Japan from 1983 to 1996 (22). The mean age at diagnosis of patients with tumors with no mutation, 49.7 years, was significantly younger than that in the control cases, 54.2 years ($P = 0.0076$). In the histologic subtypes, there was a significantly lower proportion of tumors with mucinous adenocarcinoma in the no mutation cases than in the control cases ($P = 0.038$). Although ~80% of the histologic types of *BRCA*-associated tumors were related to serous adenocarcinoma (Sekine *et al.*, manuscript in preparation) (13), the proportion of tumors with serous adenocarcinoma in the no mutation groups tended to be higher than those in the control groups, but not statistically significant ($P = 0.051$). No difference was seen in the stage distribution between the tumors with no mutation and those of the controls.

Genome-wide linkage analysis

Among the 37 families with no mutation of *BRCA1* or *BRCA2*, we performed genome-wide linkage analysis in 28 families with 58 affected patients and with nine unaffected members. Nine families were excluded from this analysis because they consisted of only mother–daughter affected pairs. Table 2 represents the details of the 28 families analyzed. Sister–sister relationships were the most common and accounted for 48 cases among 24 families. Aunt–niece relationships consisted of four cases in two families. Sister–sister–niece relationships involved three cases in one family. Niece–aunt–cousin

Table 1. Clinical and pathological characteristics of familial and sporadic ovarian cancer

	No. of cases (%)		
	Familial (no mutation)	Population controls	*P*-value
Total	78	1299	
Age (years)			
Mean ± SD	49.7 ± 9.6	54.2 ± 13.5	0.0076
Range	25–68	12–94	
Histology			
Serous	40 (54.8)	524 (44.3)	NS
Endometrioid	8 (11.0)	157 (13.3)	NS
Mucinous	10 (13.7)	273 (23.1)	0.038
Clear cell	11 (15.1)	164 (13.9)	NS
Others	4 (5.5)	66 (5.6)	
Unknown	5	115	
Stage			
I	25 (45.5)	553 (43.1)	NS
II	9 (16.4)	167 (13.0)	NS
III	13 (23.6)	434 (33.9)	NS
IV	8 (14.5)	128 (10.0)	NS
Unknown	23	17	

NS, not significant.

relationships consisted of three cases in one family. We initially screened the whole genome, including the X chromosome, by a multipoint non-parametric method using the GENEHUNTER program (23). As a result, suggestive linkage [non-parametric LOD (NPL) ≥ 2.2] was detected in only one region, chromosome 3p22–p25 (NPL = 2.77 at *D3S3611*), in the genome scan (Fig. 1).

Subsequently, multipoint parametric analyses were conducted to assess whether a dominant or recessive model could be adapted for the novel susceptibility gene. Based on the best fitting dominant gene from the result of our segregation analysis (data not shown), we obtained a heterogeneity LOD (hLOD) score of 3.49 and a homogeneity LOD (LOD) score of 3.49 on 3p22–p25. On the other hand, based on the recessive gene from the result of the described segregation analysis (21), we obtained a hLOD score of only 1.41 and a LOD score of –0.086 at the region.

The results of the two-point non-parametric and parametric analyses on 3p with GENEHUNTER, employing 28 markers spanning from *D3S1297* (3pter) to *D3S3518* (3p21), showed a *P*-value of <0.05 in eight markers and a suggestive score for linkage at two markers (NPL = 2.60 and 2.49 at *D3S1597* and *D3S3611*, respectively; Table 3). The result of a *P*-value of <0.05 at 10 markers within ~19cM (16.5–35.8 cM) was evidenced by the SIBPAL program (24).

Table 2. Characteristics of ovarian cancer families in linkage analysis

Relationship	No. of cases	No. of families
Sister/sister	48	24
Aunt/niece	4	2
Sister/sister/niece	3	1
Niece/aunt/cousin	3	1
Total	58	28

Loss of heterozygosity (LOH) analysis

For further experiments, we performed LOH analysis in an attempt to determine whether the candidate gene on 3p22–p25 contributed to the tumorigenesis as the tumor suppressor gene or in the hope of narrowing the candidate region obtained by linkage analysis. LOH analyses with the 28 markers, located from *D3S1297* to *D3S3518*, was performed on all available tumor samples, which included 50 samples with no mutation in 28 families and 58 samples with the *BRCA1* mutation in 39 families (Table 4). As a result, at the four markers in the 3p22–p25 region, *D3S3591*, *D3S3611*, *D3S3610* and *D3S1554*, LOH was detected in >50% of informative samples of patients with no mutation. However, the frequency of LOH at these four markers was decreased in tumor samples with the *BRCA1* mutation (50.0% versus 22.9%, 51.6% versus 31.7%, 52.6% versus 24.0% and 52.9% versus 40.0%, respectively). In addition, high frequency of LOH at one marker, existing at the edge of chromosome 3p near the telomere, was observed in both samples with or without the *BRCA1* mutation (50.0% and 53.8%).

Subsequently, we performed microsatellite instability (MSI) analysis on 50 tumor samples from patients without mutation, employing the same 28 markers used in the LOH analysis, to examine the probability of mismatch repair genes associated with MSI contributing to the generation of familial ovarian cancer, since one of the genes, *MLH1*, had been reported to be

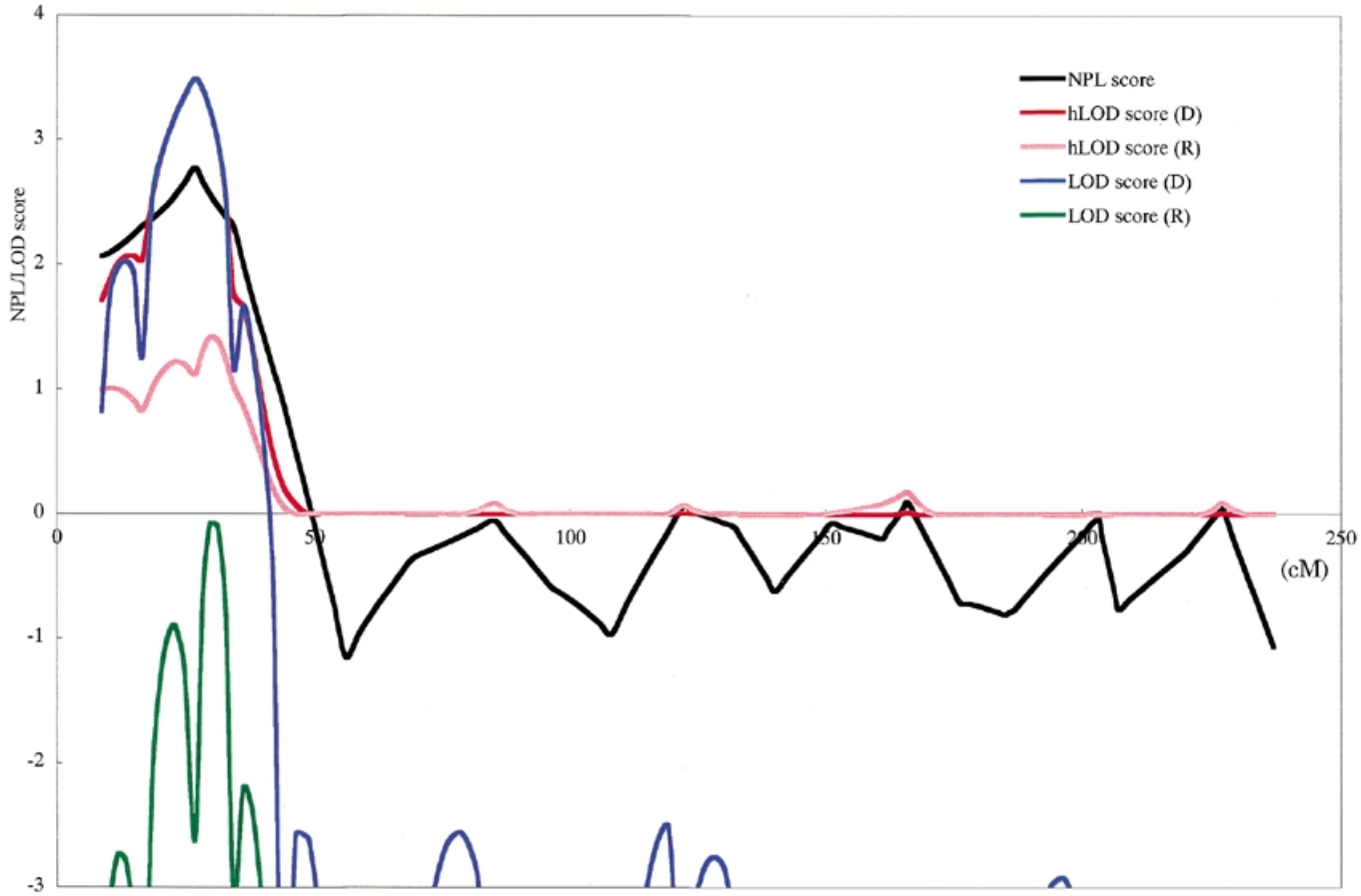

Figure 1. Multipoint non-parametric and parametric analyses for chromosome 3 from a genome-wide scan using GENEHUNTER. The *x*-axis represents the genetic distance along the chromosome in cM from the 3pter and the *y*-axis is used to depict the NPL score (black), the heterogeneity LOD (hLOD) score (red and pink) and the homogeneity LOD (LOD) score (blue and green). D, assumed the best fitting dominant model by the result of segregation analysis; R, assumed the recessive model by the result of the described segregation analysis (21). We obtained an NPL score of 2.77 (black), a hLOD score (D) of 3.49 (red), a hLOD score (R) of 1.41 (pink), a LOD score (D) of 3.49 (blue) and a LOD score (R) of –0.086 (green) on 3p22–p25.

located at 3p21. Less than 20% of the tumor samples revealed MSI for all the analyzed markers.

DISCUSSION

In 37 of the 81 ovarian cancer families, we could not detect germline mutation of *BRCA1* or *BRCA2* based on a direct sequencing method. Although it has been reported that no germline mutation of *BRCA1* or *BRCA2* was found in substantial numbers of ovarian cancer families, whether the other gene contributes to the generation of these familial ovarian cancer remains controversial. Gayther *et al.* (12) suggested that a combination of chance clustering of sporadic cases and insensitivity of mutation detection might have accounted for the remaining families. It appeared to be unlikely that the 28 families examined in this study were occasionally a clustering of sporadic cases judging from the clinical aspects, which involved the following: (i) the younger mean age at diagnosis of familial ovarian cancer patients with no mutation and (ii) the fact that the lifetime risk of ovarian cancer for Japanese women is three or four times lower than that in the USA (25). Also, the differences between ovarian cancer families with no mutation and those with the mutation of *BRCA1* or *BRCA2* could be considered. First, there is very little chance of including breast cancer patients in ovarian cancer families with no mutation compared with those with the mutation of *BRCA1* or *BRCA2* (6/37 = 16.2% versus 20/44 = 45.5%). Secondly, the number of affected members in ovarian cancer families with no mutation were fewer compared with those with the mutation of *BRCA1* or *BRCA2* (2.16 versus 2.73; Table 5), suggesting that the penetrance of the novel gene, if it exists, may be relatively low in comparison with that of *BRCA1*, whose penetrance rate in Japanese familial ovarian cancers was preliminarily calculated to be high; ~79% (13). In addition, insensitivity of mutation detection could be related to the technical variation of detecting the mutation of *BRCA1* or *BRCA2*. In the current experiments, a direct sequencing method of the entire exons, including the intronic boundary regions, was employed, although several other institutes carried out detection analysis based on single-strand conformation polymorphism and/or protein truncation test. In fact, we could find three missense mutations in 39 families with the *BRCA1* mutation (Sekine *et al.*, manuscript in preparation). Therefore, as these findings raise the possibility that additional susceptibility genes for

Table 3. Two-point linkage analyses with dense markers at candidate region on 3p

Locus	Position (cM)	GENEHUNTER				SIBPAL
		LOD score	hLOD score	NPL score	*P*-value	*P*-value
D3S1297	2.5	–20.5294	–0.0012	–0.4940	0.6883	0.7893
D3S3525	3.1	–8.3380	0.0725	0.2615	0.3988	0.5993
D3S3630	4.9	–7.3938	0.3646	0.7929	0.2154	0.3655
D3S1620	8.7	–2.3128	1.1065	1.3733	0.0852	0.0612
D3S1560	12.9	–25.2070	–0.0008	–0.8776	0.8095	0.8483
D3S1304	**16.5**	–2.3430	1.1033	1.5892	0.0561	**0.0191**
D3S3591	19.0	–10.3357	0.7801	1.4464	0.0754	0.0973
D3S3691	**23.2**	1.7269	1.7253	1.5951	0.0558	**0.0266**
D3S1597	**24.1**	3.2076	3.2073	2.6036	**0.0045**	**0.0001**
D3S3611	**26.7**	3.1739	3.1735	2.4858	**0.0063**	**0.0020**
D3S3589	**26.7**	–1.7828	1.5367	1.8182	**0.0346**	**0.0256**
D3S3693	30.4	–10.5143	0.6706	1.3609	0.0877	0.1294
D3S3714	30.9	0.9244	0.9194	0.9974	0.1605	0.1320
D3S1263	**30.9**	–1.5393	1.5803	1.9722	**0.0244**	**0.0284**
D3S3680	**30.9**	–1.9176	1.4466	1.8296	**0.0337**	**0.0222**
D3S1259	30.9	0.5889	0.5967	0.8007	0.2139	0.1605
D3S3610	31.4	0.3733	0.4728	0.7847	0.2176	0.2001
D3S1585	**33.0**	2.0557	2.0547	1.8932	**0.0294**	**0.0083**
D3S3608	**33.5**	–1.3734	1.6389	2.1019	**0.0177**	**0.0023**
D3S1554	35.7	0.3780	0.5003	0.7612	0.2235	0.1434
D3S1286	**35.8**	–1.7709	1.5676	1.9585	**0.0251**	**0.0116**
D3S1293	36.9	–3.2542	0.5098	1.0380	0.1502	0.0834
D3S1599	38.9	–2.2836	0.0030	0.2145	0.4134	0.3265
D3S3659	40.7	–7.4673	0.0898	0.3940	0.3468	0.1740
D3S2336	43.2	–14.6197	0.3590	1.1714	0.1218	0.1227
D3S2337	45.0	–5.1279	–0.0033	–0.0664	0.5253	0.3994
D3S1283	46.8	–15.5727	0.0707	0.5355	0.2977	0.1653
D3S3518	55.4	–34.3256	–0.0003	–1.5536	0.9400	0.8960

Data in bold typeface represent a *P*-value <0.05.

familial ovarian cancer are expected to exist, we performed genome-wide linkage analysis for further investigation to identify novel susceptibility genes.

Since the mode of inheritance for familial ovarian cancer is still controversial (19–21), we initially screened the whole genome by a multipoint non-parametric method with the GENEHUNTER program (23). In our genome scan, only one locus, chromosome 3p22–p25, showed evidence of a suggestive linkage. In addition, the results of further experiments in this region using dense microsatellite markers will verify the reliability of the candidate on the 3p22–p25 region obtained by screening with multipoint non-parametric analysis.

One disadvantage of the non-parametric linkage analysis which has been pointed out is that numerous affected pairs are necessary for sufficient analysis, and that the rate of false-positivity is high compared with classical parametric analysis. In our experiments, the suggestive score was obtained based on a relatively limited number of families. This could be explained by the following: First, families associated with the *BRCA1* or *BRCA2* mutation were excluded from the analyzable data, and secondly, Japanese are a relatively uniform population genetically. In addition, as we preliminarily determined allele frequencies of all markers in Japanese women, the possibility of obtaining a false-positive score appeared to be low.

Several tumor suppressor genes are reported to exist around the 3p22–p25 region, such as *VHL* (OMIM: 193300) and *XPC* (OMIM: 278720) located at 3p25, *TGFBR2* (OMIM: 190182) at 3p22, and *MLH1* (OMIM: 120436) at 3p21 (Fig. 2). However, one could eliminate the possibility that the additional tumor suppressor gene on 3p22–p25 for familial ovarian cancer is identical to these genes. This must be true because

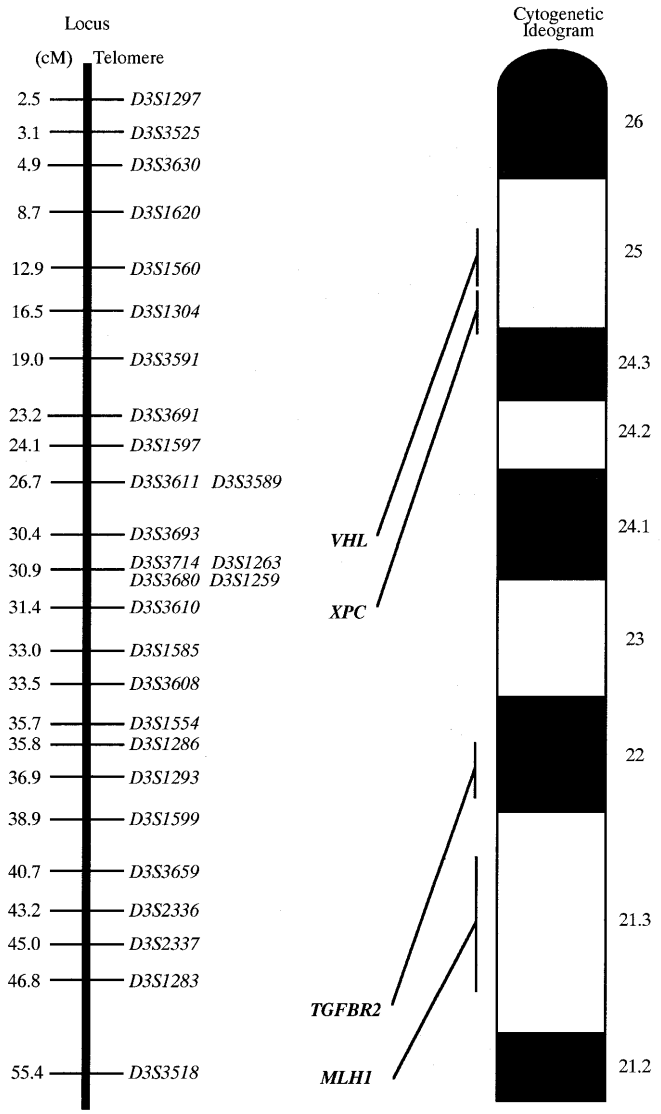

Figure 2. Summary of microsatellite markers used in the analysis and regions of interest on chromosome 3p. Genetic markers used in the analysis are listed in descending order from the 3pter to 3p21 according to recent websites (http://www.ncbi.nlm.nih.gov/genemap/ and http://www.ncbi.nlm.nih.gov/Entrez/). The approximate physical maps of markers and genes, *VHL*, *XPC*, *TGFBR2* and *MLH1* are also shown.

there is no evidence for an excess risk of epithelial ovarian cancer in carriers with mutation of *VHL*, *XPC* or *TGFBR2* (26–30). In addition, as a result of the MSI analysis, the possibility of the contribution of *MLH1* in the 28 families analyzed is unlikely. Several other genes mapped to this region, such as *OGG1* (8-oxoguanine DNA glycosylase, OMIM: 601982) and *RAF1* (v-raf-1 murine leukemia viral oncogene homolog 1, OMIM: 164760) at 3p25, *TOP2B* (topoisomerase II β, OMIM: 126431) and *PCAF* (p300/CBP-associated factor, OMIM: 602303) at 3p24, and *RAB5A* (member of the RAS oncogene family, OMIM: 179512) at 3p22–p24, may be implicated in the process of carcinogenesis, so these genes possess the probability of being candidate genes for familial ovarian cancer.

As a result of the LOH analysis, high frequency of LOH was observed in both no mutation and *BRCA1* mutation groups at *D3S1297* at the end of chromosome 3p. It has been documented that the end of the chromosome arm close to the telomere was more unstable than the other sites, suggesting the reliability of the results. In the present experiment, only modest evidence for ovarian cancer susceptibility locus at 3p22–p25 was obtained, and no direct evidence to specify the candidate region in the locus was obtained based on the LOH analysis. Nevertheless, the investigation to identify a novel susceptibility gene in Japanese ovarian cancer families is advantageous in that positional cloning could be successfully performed on a limited number of families, since Japanese consist of a more homogeneous population racially than people in Western countries. In addition, the prospect of a project, such as an association study with more precisely designed microsatellite and/or single nucleotide polymorphism markers,

Table 4. Loss of heterozygosity at candidate region on 3p

Locus	No mutation		*BRCA1*	
	No. of LOH cases[a]	No. of MSI cases[a]	No. of LOH cases[a]	*P*-value[b]
D3S1297	8/16 (50.0)	0	14/26 (53.8)	NS
D3S3525	11/29 (37.9)	3/32 (9.4)	13/28 (46.4)	NS
D3S3630	9/36 (25.0)	0	15/45 (33.3)	NS
D3S1620	11/36 (30.6)	0	12/43 (27.9)	NS
D3S1560	7/18 (38.9)	2/20 (10.0)	16/38 (42.1)	NS
D3S1304	14/36 (38.9)	1/37 (2.7)	14/44 (31.8)	NS
D3S3591	12/24 (50.0)	1/25 (4.0)	8/35 (22.9)	0.030
D3S3691	10/26 (38.5)	2/28 (7.1)	10/32 (31.3)	NS
D3S1597	6/23 (26.1)	2/25 (8.0)	9/30 (30.0)	NS
D3S3611	16/31 (51.6)	5/36 (13.9)	13/41 (31.7)	NS (0.071)
D3S3589	11/34 (32.4)	0	15/46 (32.6)	NS
D3S3693	11/32 (34.4)	4/36 (11.1)	9/38 (23.7)	NS
D3S3714	9/23 (39.1)	0	8/30 (26.7)	NS
D3S1263	9/23 (39.1)	5/28 (17.9)	17/38 (44.7)	NS
D3S3680	11/26 (42.3)	2/28 (7.1)	13/39 (33.3)	NS
D3S1259	8/26 (30.8)	6/32 (18.8)	11/35 (31.4)	NS
D3S3610	10/19 (52.6)	0	6/25 (24.0)	0.051
D3S1585	10/36 (27.8)	6/42 (14.3)	15/39 (38.4)	NS
D3S3608	9/25 (36.0)	5/30 (16.7)	17/36 (47.2)	NS
D3S1554	9/17 (52.9)	1/18 (5.6)	10/25 (40.0)	NS (0.30)
D3S1286	10/40 (25.0)	2/42 (4.8)	18/47 (38.3)	NS
D3S1293	12/32 (37.5)	1/33 (3.0)	14/40 (35.0)	NS
D3S1599	12/31 (38.7)	0	11/26 (42.3)	NS
D3S3659	8/30 (26.7)	0	12/30 (40.0)	NS
D3S2336	7/36 (19.4)	2/38 (5.3)	16/40 (40.0)	0.044
D3S2337	10/35 (28.6)	8/43 (18.6)	12/36 (33.3)	NS
D3S1283	14/35 (40.0)	1/36 (2.8)	14/39 (35.9)	NS
D3S3518	11/29 (37.9)	3/32 (9.4)	12/28 (42.9)	NS

LOH, loss of heterozygosity; MSI, microsatellite instability; NS, not significant.
[a]No. of informative cases (percentages in parentheses).
[b]No mutation versus *BRCA1* (Fisher's exact test).

or expression analysis of expressed sequence tag and known genes located at the 3p22–p25 region, offers an attractive model for further investigation.

MATERIALS AND METHODS

Families

We ascertained 196 epithelial familial ovarian cancer patients in 81 ovarian cancer families in Japan. The criterion for an ovarian cancer family involved two or more members with well documented epithelial ovarian cancer in the second degree relatives. We examined the clinical data from hospital records and pathological reports, or asked physicians to answer questionnaires or hear from patients, and confirmed that all affected individuals were primary epithelial ovarian cancer All experiments were performed under informed consent.

Mutational analysis for *BRCA1* and *BRCA2*

Direct sequencing. A gemonic DNA was prepared from lymphocytes and paraffin-embedded block using the standard phenol/chloroform methods. We performed direct sequencing of available patients with ovarian cancer in all 81 families. The entire exons, 23 in *BRCA1* and 26 in *BRCA2*, and the intronic boundary regions were sequenced in both forward and reverse directions for detecting germline mutations. The non-coding intronic regions that were analyzed did not extend >20 bp proximal to the 5′ end and 10 bp distal to the 3′ end of each exon. These regions were amplified by PCR respectively from

Table 5. Details of ovarian cancer families by number of affected members and results of *BRCA1, 2* mutational analysis

No. of ovarian cancer cases in a family	No. of families		
	BRCA1	*BRCA2*	No mutation
2	20	5	32
3	11	0	4
4	4	0	1
5+	4	0	0
Total	39	5	37

100 ng of genomic DNA (35 reactions for *BRCA1* and 47 reactions for *BRCA2*). The PCR products were sequenced by the dideoxy method using an Autocycle sequencing kit (Pharmacia Biotech in Japan, Tokyo) and end-labeled by Cy5 primer. PCR products were electrophoresised in a 6% polyacrylamide gel and analyzed with an automatic sequencer, ALF express (Pharmacia Biotech).

Statistical analysis. Clinical characteristics among ovarian cancer patients were tested by unpaired *t*-test, χ^2 analysis and Fisher's exact test.

Genome-wide linkage analysis

Genotyping. We used 410 microsatellite markers on the basis of the Genethon map (32). The average intermarker distance was 9.0 cM. One of the pairs of the PCR primers was end-labeled by Cy5. PCR amplification using 25 ng of DNA was carried out, and after mixing with 95% formamide and denaturation, the products were resolved by electrophoresis in 6% polyacrylamide gels and analyzed on the autosequencer, ALF express (Pharmacia Biotech). Allele assignment was performed by Fragment manager software for comparison of CEPH family members 134702. The interpretation of alleles was checked by two different individuals to verify Mendelian segregation prior to computer processing. The frequencies of the alleles of each marker were determined by DNA typing of 35 normal Japanese women with no family history of cancer (data not shown). Some of the markers which did not yield satisfactory results after two PCRs and gel electrophoresis and whose heterozygosity was <60% were replaced by additional linked markers from the Genethon map (31).

Statistical methods. We used two different computer programs for linkage analysis. For non-parametric and parametric analyses, the GENEHUNTER program (version 2.1) was employed (23). GENEHUNTER estimates the statistical significance of sharing alleles identical-by-descent between all affected individuals, as well as how much of the total genetic information in a segment has been extracted from the markers studied. On the other hand, for non-parametric two-point analyses, we used the SIBPAL program from the SAGE package. SIBPAL is based on methods first proposed by Haseman and Elston (24). This program is available for not only affected sib-pair analysis, but also for affected–unaffected sib-pair analysis. For both types of analyses, we set the genome-wide false positive rate at 5% and used established criteria (32). Linkage evidence at a single point in the genome is considered significant whenever the *P*-value is $\leq 2.2 \times 10^{-5}$ and/or the NPL score is ≥3.6. The evidence is considered suggestive whenever the *P*-value is between 2.2×10^{-5} and 7.4×10^{-4} and/or the NPL score is between 2.2 and 3.6.

LOH analysis

We analyzed the samples from 50 cases with no mutation in 28 families included in the linkage study and from 58 cases with *BRCA1* mutation in 39 families. PCR amplification of microsatellite repeat polymorphisms was used for the detection of LOH. Twenty-eight markers spanning the regions of interest on 3p were selected for use. PCR products from normal DNA and those from tumor DNA were compared using an autosequencer. Normal DNA and tumor DNA samples were electrophoresed at the same time. LOH was scored based on the absence, or a difference in the relative intensity, of alleles in the tumor compared with normal DNA. A decrease of >50% of the intensity of the bands in a tumor sample compared with normal DNA was determined as the LOH.

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