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Original Paper

Expression of Abnormal Transcripts of the *FHIT* (Fragile Histidine Triad) Gene in Ovarian Carcinoma

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To elucidate the role of the *FHIT* (fragile histidine triad) gene in ovarian carcinogenesis, the expression of the gene was analysed by reverse transcription–polymerase chain reaction (RT–PCR) in 51 cases of ovarian carcinoma, 6 cases of borderline tumour and 4 cases of benign ovarian tumour. The concomitant expressions of normal and abnormal FHIT transcripts were detected in 39% of carcinomas and in 83% of borderline tumours, while benign tumours and normal ovarian tissues expressed only normal transcript. In addition, there were 4 (8%) cases of carcinoma lacking expression of normal FHIT transcript, all of which were in advanced stages (stage III-IV) and poorly differentiated. These results suggest that the expression of abnormal transcripts of the *FHIT* gene is a feature of ovarian malignant/borderline tumours and that the complete loss of normal FHIT expression is related to the progression of ovarian carcinoma in a subset of the cases. However, abnormal FHIT transcripts themselves were not associated with any clinicopathological parameters, such as clinical stage, histological subtype of tumour, grade of differentiation or outcome of the patient. Additionally, abnormal FHIT expression was not associated with the presence of loss of heterozygosity (LOH) at this locus, suggesting that abnormal FHIT transcripts are not derived from genetic alteration or that genetic alteration at this locus is complicated. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: FHIT, ovary, carcinoma, mRNA, deletion
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INTRODUCTION

OVARIAN CARCINOMA is the leading cause of death from malignancies of the female genital tract. Various genetic events have been implicated in the development of ovarian carcinoma, which include amplification and overexpression of the c-*erb*B-2 gene [1], mutational activation of the K-*ras* gene [2] and mutation of the *p53* tumour suppressor gene [3]. In addition, analysis of loss of heterozygosity (LOH) revealed that various chromosomal loci, including chromosome 3, are associated with ovarian carcinogenesis, suggesting the presence of putative suppressor genes [4, 5]. The *FHIT* (fragile histidine triad) gene has recently been identified as a candidate tumour suppressor gene at the 3p14.2 locus by positional cloning [6]. This locus is considered a suppressor locus because reciprocal t(3;8)(p14.2; q24) translocation has been reported in familial renal cell carcinoma [7] and an aphidicolin-inducible fragile site, FRA3B, was found to be indistinguishable from this t(3;8) translocation [8]. Subsequent analysis revealed that various malignant tumours, including carcinomas of lung, head and neck [9–11], Merkel cell carcinoma [12], colorectal carcinoma [6, 13] and breast carcinoma [14], expressed aberrant FHIT transcripts derived from splicing variation, homozygous deletion or rearrangement. FHIT protein is structurally considered a member of the histidine triad (HIT) gene family [6]. Recently, it has been reported that FHIT protein has the enzymatic activity of a diadenosine 5′, 5‴-P^1, P^4-triphosphate (Ap_3A) hydrolase and that loss of this enzymatic activity, resulting in the elevation of Ap_3A, may contribute to carcinogenesis [15]. Although the significance of abnormal transcripts demonstrated in various malignant tumours is unclear, it is suggested that hampered production of normal FHIT protein is associated with tumour development [14, 15].

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To elucidate the role of FHIT in the development of ovarian carcinoma, we analysed the structure of FHIT transcripts by reverse transcription–polymerase chain reaction (RT–PCR) and by sequencing in epithelial ovarian tumours as well as in normal ovarian samples. The relationship between the status of FHIT transcription and various clinicopathological parameters was also evaluated. In addition, the structure of the *FHIT* gene was examined using microsatellite markers at the 3p14 locus.

MATERIALS AND METHODS

Tissue samples and cell lines

Fresh surgical specimens of ovarian tumours were obtained from patients who underwent oophorectomy and hysterectomy. There were 51 cases of epithelial ovarian carcinoma, 6 cases of borderline tumours and 5 cases of benign epithelial tumours. 4 cases of normal ovarian tissue were also obtained. All specimens were used for investigation after written informed consent. The tissues were prepared carefully under a dissecting microscope to eliminate inappropriate components, such as stroma or necrotic tissues, and stored at − 80°C for subsequent analysis. If necessary, tumour tissues were obtained from collected 10 μm thick frozen sections, one of which showed that the tumour component exceeded 80%. Clinical staging was performed according to the classification of the International Federation of Obstetrics and Gynecology (FIGO, 1987) (Table 1). The patients were followed for 1–60 months (median follow-up period 15 months). Two cell lines derived from ovarian adenocarcinomas, SK-OV-3 and NIH: OVCAR-3, were purchased from the American Type Culture Collection.

RNA preparation and RT–PCR

Isolation of total RNA and preparation of complementary DNA were carried out according to the methods previously described [16]. The nested-PCR amplification of the *FHIT* gene was essentially the same as that of Sozzi and colleagues [9], using the primers 5U2/3D2 and 5U1/3D1 described previously. As a control, β_2-microglobulin transcript was amplified from the same cDNA [16]. Aliquots of PCR products were electrophoresed in 1% agarose gels and visualised by ethidium bromide staining.

Sequencing analysis

In cases with abnormal FHIT transcript, PCR products were electrophoresed either in 6% acrylamide gels or in SeaKem GTG agarose gels (FMC Bioproducts, Rockland, Maine, U.S.A.) and abnormal bands were recovered using EASYTRAP version 2 (Takara Shuzo, Ohtsu, Shiga, Japan) according to the manufacturer's protocol. Cycle sequence analysis was performed with a PRISM cycle sequencing kit (Applied Biosystems, Foster City, California, U.S.A.) and analysed with an autosequencer, ABI 373 STRETCH (Applied Biosystems).

Analysis of LOH at the 3p14 locus

Three microsatellite markers at 3p14 were used for LOH analysis. Forty cycles of PCR amplification were performed using primers according to the Genome Database. Aliquots of products were mixed 1:1 with 95% formamide loading buffer and electrophoresed in 6% denaturing acrylamide gels containing 8 M urea. Gels were then stained with a 1:10000 dilution of SYBR Green I (Molecular Probes, Eugene, Oregon, U.S.A.) and analysed with a FluorImager SI (Molecular Dynamics, Tokyo, Japan).

Statistical analysis

The relative frequency between two groups was compared using the chi-squared test. The prognosis of two groups was compared using generalised Wilcoxon's analysis.

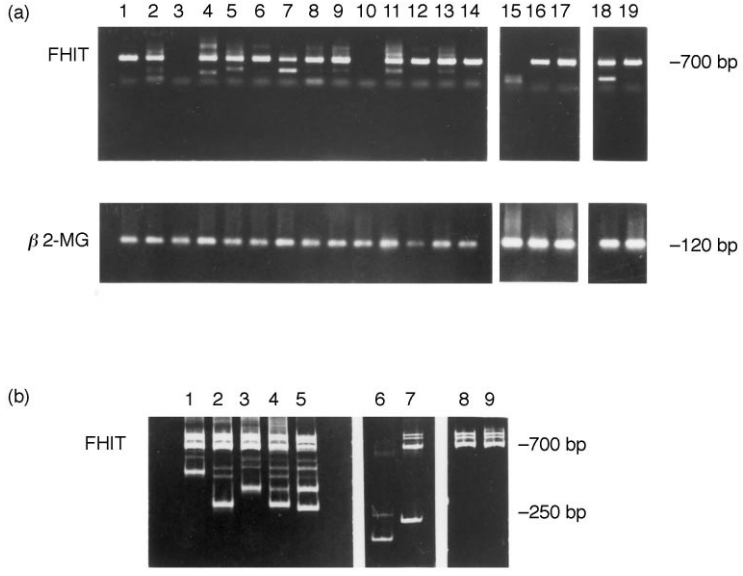

Figure 1. Expression of the FHIT transcript as detected by reverse transcription–polymerase chain reaction (RT–PCR). (a) Detection in agarose gel electrophoresis (top). Lanes 1—16 and 18–19 correspond to cases 18, 28, 26, 47, 55, 38, 52, 57, 39, 45, 32, 25, 8, 51, 37, 44, 21 and 9, respectively, in Table 1. Lane 17 is a sample from normal ovary. One to two abnormal transcripts are detected in some lanes. Expression of β_2-microglobulin as a internal control is also shown. (b) Detection in acrylamide gel electrophoresis. Lanes 1–8 correspond to cases 43, 21, 31, 1, 4, 37, 2 and 9, respectively, in Table 1. Lane 9 is a sample from normal ovary. In lanes 1–5 and 7, two to four minor (thin) bands were detected along with one to two major bands. Case 37 in lane 6 shows only abnormal transcript.

Table 1. *The age, clinical stage, histology and expression pattern of the FHIT gene loss of heterozygosity (LOH) at the* 3*p*14 *locus in ovarian carcinoma cases*

Case	Age (years)	Stage	Histology	Grade	FHIT	Sequence	13pLOH
1	51	I	Serous	1	†	L, A, B, C, D, G	
2	56	I	Serous	1	†		
3	78	I	Serous	1	⋆	L	
4	43	I	Endometrioid	1	†	L, A, B, C, G	
5	45	I	Endometrioid	2	†		P
6	51	I	Endometrioid	3	⋆		N
7	59	I	Endometrioid	1	⋆		
8	60	I	Endometrioid	2	†		
9	43	I	Endometrioid	3	⋆	L	
10	47	I	Clear cell	2	⋆		N
11	52	I	Clear cell	3	†		N
12	40	I	Clear cell	1	†		N
13	54	I	Clear cell	1	⋆		
14	49	I	Clear cell	2	⋆		
15	72	I	Clear cell	2	⋆		
16	54	II	Serous	1	†		
17	70	II	Endometrioid	2	⋆		
18	47	II	Clear cell	2	⋆		P
19	40	III	Serous	2	⋆		P
20	46	III	Serous	3	⋆		
21	47	III	Serous	3	†	L, B, C, D	
22	48	III	Serous	2	†		
23	54	III	Serous	2	⋆		
24	58	III	Serous	2	⋆		
25	61	III	Serous	2	⋆		N
26	63	III	Serous	3	‡		N
27	63	III	Serous	1	⋆		
28	67	III	Serous	3	†		
29	48	III	Serous	3	⋆		
30	26	III	Serous	1	†		
31	52	III	Serous	ND	†	L, A, B, G	
32	67	III	Serous	2	†	L, A	
33	67	III	Serous	2	†		
34	66	III	Serous	2	⋆		N
35	24	III	Serous	1	⋆		
36	58	III	Serous	2	⋆		
37	57	III	Serous	2	‡	F	
38	58	III	Serous	3	⋆		
39	40	III	Serous	1	†		
40	45	III	Endometrioid	3	⋆		P
41	49	III	Endometrioid	3	‡	G	N
42	62	III	Endometrioid	1	†		
43	46	III	Clear cell	1	†	L, B, G	
44	30	IV	Serous	2	⋆		
45	62	IV	Serous	3	‡		P
46	61	IV	Serous	ND	⋆		
47	19	IV	Serous	2	†		
48	49	IV	Serous	2	⋆		
49	46	IV	Serous	2	†		
50	67	IV	Serous	1	⋆		
51	47	IV	Mucinous	3	⋆		
52	55	I	Mucinous		†		
53	60	I	Mucinous		†		
54	64	I	Mucinous		†		
55	52	I	Mucinous		†		
56	42	I	Serous		⋆		
57	28	I	Serous		†		

⋆cases expressing only normal transcript; †cases expressing both normal and abnormal transcripts; ‡cases lacking expression of normal FHIT transcript. L and A–G correspond to full length mRNA fragment and variant mRNA A–G, respectively, which are indicated in Figure 2(a). No FHIT expression was detected in cases 26 or 45, while only abnormal transcript was expressed in cases 37 and 41. Grade, histological grade of differentiation; P, positive; N, negative. Cases 52 to 57 represent borderline tumours.

RESULTS

Expression of abnormal FHIT transcripts in ovarian carcinoma

Of 51 cases of ovarian carcinoma, 27 (53%) expressed only normal FHIT transcript, while 20 (39%) cases showed simultaneous expression of normal and abnormal transcripts, and the remaining 4 (8%) cases did not express normal FHIT transcript. 2 cases of the last group did not express any FHIT transcript, while the other 2 cases expressed only abnormal transcript (Table 1, Figure 1a). Both ovarian cancer cell lines, OVCAR-3 and SKOV-3, expressed both normal and abnormal transcripts. In borderline tumours, 5 of 6 (83%) cases showed normal and abnormal FHIT transcripts, whilst one had only normal FHIT expression. In contrast, all 5 epithelial benign tumours and 4 normal ovarian tissues expressed only normal FHIT transcript. In cases with abnormal FHIT transcript, the PCR products were electrophoresed either in 6% acrylamide gels or in SeaKem GTG agarose gels. This showed that there were one to seven faint (minor) bands in addition to the one to three major bands easily detected in normal agarose gel electrophoresis (Figure 1b). These minor bands were not observed when samples from cases with only normal transcript were examined with acrylamide gels.

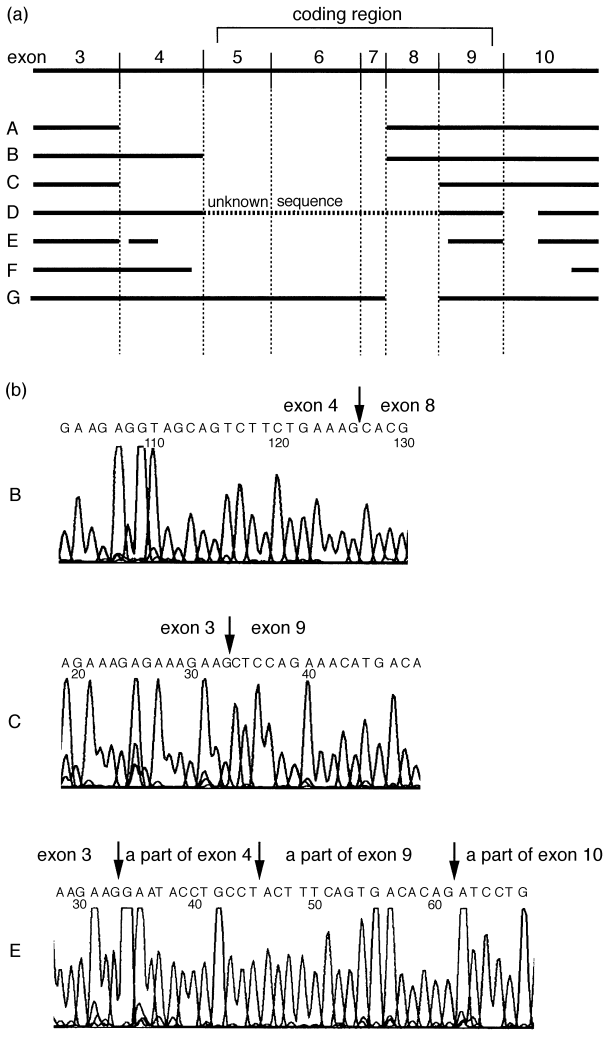

Figure 2. Sequence analysis of an abnormal transcript of the *FHIT* gene. (a) Schematic structure of truncated variants of FHIT transcript. All the variants lacked either exon 5 or 8 or both. (b) Representative sequence of abnormal FHIT transcript determined by an automated sequencer. Arrows indicate abnormal junction of truncated transcripts. B, C and E correspond to species of the abnormal transcript indicated in (a).

Sequence analysis of FHIT transcript

The sequence of abnormal transcript was obtained from 10 carcinomas including 2 cases lacking normal FHIT transcripts. All the abnormal transcripts lacked several exons, including either exon 5 (exons 4–7, 4–8, 5–7) or exon 8 (exons 4–8, exon 8), consistent with previous reports (Figure 2a, A–C, G, Figure 2b, B, C). A more complicated pattern was observed in some transcripts, in which parts of the exons were combined (Figure 2a, E, F, Figure 2b, E) or an unknown sequence was inserted (Figure 2a, D). These complicated structures were not the result of non-specific amplification of PCR, because completely identical products (for example, product E in Figure 2a, b) were sequenced in some cases. Notably, most of the minor (faint) bands had the same sequence as the major bands, suggesting that they are not the non-specific products of PCR but splicing variants.

Analysis of LOH at the 3p14 locus

LOH at the 3p14 locus was examined using three microsatellite markers, *D3S1234*, *D3S1300* and *D3S4301*, in 13 cases of ovarian carcinoma in which genomic DNA was obtained from both normal and carcinoma tissues (Figure 3). The LOH in at least one of these markers was detected in 5 of 13 (38%) cases of ovarian carcinoma (Table 1). There was no correlation between the presence of LOH and the expression of abnormal FHIT transcript ($P = 0.35$). 3 cases lacking normal FHIT transcript were analysed, and two of them showed LOH, while the other did not (Table 1).

FHIT expression and clinicopathological factors

Clinicopathological parameters, such as age of the patient, stage of the disease, histological subtype, grade of differentiation and outcome of the patient, were compared between cases with normal FHIT expression and cases with abnormal FHIT expression. There were no significant differences in clinicopathological parameters between these two groups (P value = 0.50, 0.85, 0.72, 0.43 and > 0.05, respectively). However, all the cases lacking normal FHIT

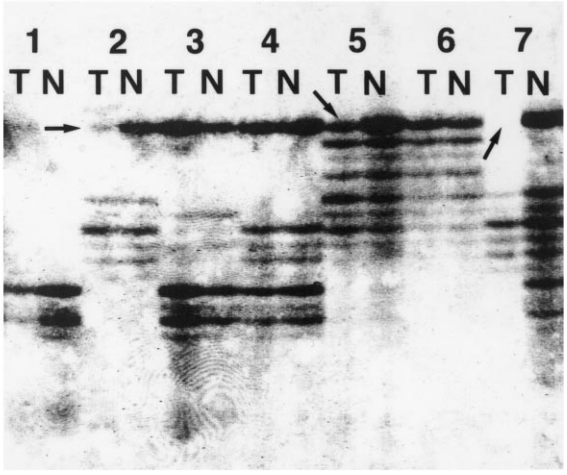

Figure 3. Detection of loss of heterozygosity (LOH) at the 3p14 locus using microsatellite marker *D3S1300*. Pairs of lanes 1–7 correspond to cases 40, 18, 25, 6, 19, 26 and 45, respectively. T, ovarian carcinoma; N, normal tissue in the same case. Arrows indicate allelic deletions.

transcript were in stage III or IV, while all the stage I or II carcinomas, including stage I borderline tumours, expressed normal transcript irrespective of abnormal transcript (Table 1). The histological grade of these tumours lacking normal transcript was relatively high (grade 3 in 3 cases and grade 2 in 1 case).

DISCUSSION

The current study demonstrated that, of 51 cases of ovarian carcinoma examined, 20 (39%) cases expressed abnormal transcripts of the *FHIT* gene and 4 (8%) cases did not express normal FHIT transcript. The frequency of abnormal FHIT expression is similar to that of head and neck squamous cell carcinoma [10] or Merkel cell carcinoma [12], less frequent than lung carcinoma [9] and more frequent than breast carcinoma [14]. 19 cases with abnormal FHIT transcript simultaneously expressed the normal transcript. This was the case in two ovarian carcinoma cell lines. Sequence analysis revealed that most of these abnormal transcripts lacked exons including exon 5 or exon 8 or both, which is in agreement with previous reports [9–12]. Interestingly, the borderline ovarian tumour expressed abnormal FHIT transcript in a significant population (83%), while all the normal ovarian tissues and benign ovarian neoplasms expressed only normal FHIT transcript. These data suggest that the abnormality in FHIT transcription is a feature of malignant and borderline epithelial ovarian tumour tissues and these abnormal transcripts originate from tumour cells, not from the contaminating normal tissue. However, the amount of abnormal FHIT transcript detected in electrophoresis was rather small compared with normal transcript in most of the ovarian carcinomas and cell lines (Figure 1a). Therefore, it is unlikely that the presence of abnormal transcript is causally linked to the relative decrease of normal FHIT transcript. A similar situation has been reported in breast cancer and malignant cell lines. Negrini and associates [14] proposed that the aberrant transcript has a dominant-negative effect against normal protein and, in this case, the relatively small fraction of abnormal FHIT protein could influence the normal FHIT function.

Analysis of clinicopathological parameters in ovarian carcinoma cases revealed that the presence of abnormal FHIT transcript itself is unlikely to affect the progression of ovarian carcinoma, unless there is a specific effect of abnormal transcript as proposed above, because no difference in clinicopathological factors was found between the groups with and without abnormal FHIT transcript. In contrast, all the cases lacking normal FHIT expression were in advanced stages and poorly differentiated, while all the early stage carcinomas expressed normal FHIT transcript. This indicates that entire loss of normal FHIT expression may play some role in the progression of ovarian carcinoma. Since FHIT protein is reported to have Ap_3A hydrolase activity, loss of this enzymatic activity resulting in elevated levels of Ap_3A is thought to contribute to carcinogenesis [15], possibly through activation of the cell cycle.

Microsatellite polymorphism analysis revealed that LOH at the 3p14 locus was present in 38% of ovarian carcinoma cases, consistent with previous reports [4, 5]. However, the presence of LOH was not significantly associated with abnormal FHIT transcription, although the sample size is rather small for statistical analysis. Explanations for the disparity between genomic and mRNA structures include the possibility that some of the abnormal PCR products are derived from non-specific amplification, since we used a nested PCR method that is susceptible to artifacts. Alternatively, abnormal FHIT transcript does not necessarily result from genetic alteration, but rather from modification of transcriptional regulation, such as the splicing mechanism in ovarian tissues. This appears to be supported by the finding that most of the cases with abnormal FHIT transcript showed more than three kinds of abnormal bands. Recently, however, Druck and colleagues [17] fully analysed the genomic structure of the *FHIT* gene in reference to aberrant mRNA, and found that complicated alterations such as discontinuous deletions, can occur in this site, resulting in an unusual pattern of FHIT transcription. Further examination of the genomic structure is necessary to understand FHIT function and events occurring in ovarian carcinoma.

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