Loss of Heterozygosity at the FHIT Gene in Different Solid Human Tumours and its Association with Survival in Colorectal Cancer Patients

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Abstract. Background: Genomic alterations and abnormal expression of the FHIT gene have been reported for a number of cancers. FHIT encompasses FRA3B, the most common fragile site in the human genome, and is suggested to be a candidate tumour suppressor gene. Materials and Methods: We analysed and compared the loss of heterozygosity (LOH) pattern in 397 solid human tumours from 9 different locations, using four polymorphic microsatellite markers within the gene (D3S1234, D3S1300, D3S2757 and D3S4260), and two markers (D3S1313 and D3S1600) flanking the gene. In addition, we tested whether there was an association between FHIT LOH and overall patient survival in colorectal cancer. Results: LOH at the FHIT gene affecting at least one of the investigated markers was detected in 166 out of 332 informative tumours, or 50%. The highest detected LOH was in lung tumours (66%) while the lowest was in thyroid and endometrium tumours (30% and 31%, respectively). Breakpoints were found inside the gene in all tumour types in 12-80% of the tumours with FHIT LOH depending on tumour type, and up to 41% could additionally be located adjacent to the 3' or 5' end of the FHIT gene. Thus we were able to locate breakpoints within or in the vicinity of the FHIT gene in 25-100% of different tumours with LOH. Although not statistically significant, we observed a trend towards a poorer survival of patients with FHIT LOH versus those with retention of heterozygosity. Conclusion: Based on our results, LOH of the FHIT gene is a common event in all tumour types analysed with a possible association with poorer survival in colorectal cancer patients. LOH at all markers analysed was, in most of the tumour types, a more common pattern of alterations than breakpoints.

FHIT (fragile histidine triad) is a putative tumour suppressor gene located on human chromosome 3p14.2. FHIT spans one of the most active common fragile sites of the human genome, the aphidicolin-sensitive site FRA3B. It contains a renal clear cell carcinoma-associated chromosomal translocation point, t(3;8) (1) and a HPV16 integration site has also been mapped within this locus (2). The 1.8 Mb FHIT gene is composed of 10 exons of which exons 5 through 9 are protein coding; it encodes a small mRNA (1.1 kb) and a small protein (16 kDa) made of 147 amino acids (1).

The human FHIT protein has enzymatic activity as a diadenosine 5'5'-P'-P'-triphosphate (ApppA) hydrolase (3), but the biological mechanism of FHIT activity and the cellular pathways associated with its tumour suppressor function are still not fully understood. Genetic, biochemical and crystallographic characterisation of FHIT suggests that the FHIT-substrate complex, rather than the hydrolase activity, is the active signalling form (4, 5).

LOH (loss of heterozygosity) is one of the most common genetic alterations involved in cancer development and is associated with the presence of tumour suppressor genes. FHIT LOH has been detected in various tumours and cancer cell lines, such as breast (6-8), thyroid (9), esophageal (10), lung (11-13), gastric (14), pancreatic (15), urinary bladder (16, 17), ovarian (18), oral squamous cell carcinomas (19, 20) and clear cell renal cell carcinoma (21). Furthermore, FHIT LOH is frequently associated with abnormal RNA expression and/or absence of protein (16, 22, 23) and cell lines from several tumour types carry homozygous deletions at the FHIT locus (1, 24-28). In general, all of the above supports the candidacy of FHIT as a tumour suppressor gene. However somatic point mutations are rare and germline mutations do not seem to be significant (28-30). The alterations detected at

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the FHIT locus could thus simply be a reflection of the unstable nature of the FRA3B region in tumour cells.

An epigenetic alteration of the gene, such as methylation, is an alternative mechanism for silencing FHIT. A high percentage of primary NSCLC (non-small cell lung cancer) samples, primary breast carcinomas, as well as lung and breast cancer cell lines, have been described with methylated FHIT and a significant correlation was found between loss of FHIT mRNA and Fhit expression (31).

Support for the role of FHIT as a tumour suppressor has also come from FHIT gene replacement and knockout experiments. Stable exogenous FHIT expression in FHIT-negative lung, gastric and renal cancer cells resulted in inhibition of tumour cell growth in nude mice (4, 32, 33). This growth inhibitory effect, observed in FHIT re-expressing tumour cell lines, has been shown to be caused by increased apoptosis and cell cycle arrest (32). Similarly, it has been demonstrated that reintroduction of FHIT protein by adenoviral-FHIT gene transduction into lung, head and neck and esophageal cancer cell lines caused apoptosis and inhibition of tumourigenicity (34, 35). Interestingly, it has been reported that apoptosis in human cultured cells is associated with a decrease of free ApppA levels (36). Therefore FHIT-induced apoptosis may be linked to its ability to reduce the intracellular level of diadenosine triphosphate, by binding and hydrolysing.

Gene knockout studies have demonstrated that both FHIT +/– and –/– mice have a higher frequency of developing spontaneous and carcinogen-induced tumours, as compared to normal FHIT +/+ littermates (37). The spontaneous tumour spectrum in the FHIT-deficient mice is different from that reported for other tumour suppressors, although it shows a partial overlap with the VHL (von Hippel Lindau) conditional knockout mice (37). The carcinogen-induced tumours in FHIT-deficient mice were, on the other hand, similar to those observed in Muir-Torre syndrome, a human cancer syndrome caused by a mismatch repair deficiency (38).

Interestingly, the frequency of either spontaneous or carcinogen-induced tumour development in FHIT +/– and –/– mice was not different, suggesting that haploid insufficiency of FHIT may promote tumour growth (37). This would explain the similar pattern of tumour spectra observed in mice with one or both FHIT alleles inactivated and the general lack of somatic point mutations and germline mutations in FHIT-associated cancers. Further support for the tumour suppressor function of FHIT was demonstrated by inhibition of tumour development by adenoviral or adenovirus-associated viral expression of the human FHIT gene in FHIT +/– mice after their carcinogen exposure (39).

In this study, by using four intragenic and two adjacent markers, we analysed and compared the FHIT LOH pattern in 397 solid tumours from 9 different locations. Furthermore, we tested whether there was an association between FHIT LOH and overall patient survival in colorectal cancer.

Materials and Methods

Patients and tumour material. Samples from 397 primary solid tumours and matching non-malignant tissue samples were obtained on the day of surgery. The samples were immediately frozen at -70°C. The tumours were from the following locations: gastric (36); colorectal (108); lung (69); endometrium (22); ovary (41); testis (30); renal (62); thyroid (14) and soft tissue sarcoma (15). All information on the tumours was recorded at the Department of Pathology, University Hospital of Iceland. Information on the patients’ date of death was recorded at the National Registry.

DNA extraction and analysis. Tumour and normal DNA was extracted from the tissue samples with proteinase K using a method developed for paraffin-embedded tissue (40). Paired tumour and normal DNA was subjected to PCR analysis. Dynazyme™ polymerase (from Finnzymes Oy, Espoo, Finland) was used in the buffer solution provided by the manufacturer. Samples were subjected to 35 cycles of amplification, consisting of 30 seconds at 55°C, 60 seconds at 72°C and 30 seconds at 95°C, which was followed by 10 minutes at 72°C. Four of the markers used are located within the FHIT gene; D3S1234 and D3S1300 in intron 5; D3S2757 and D3S4260 in intron 4. In addition, we used two markers flanking the gene, D3S1313 which is located telomeric to exon 10 and D3S1600 centromeric to exon 1. The markers were obtained from TAG Copenhagen (AS Symbion, Fruebjergvej 3 DK-2100 Copenhagen, Denmark). To facilitate binding, primers were elongated by terminal-transferase to 40 μM K-HEPES/1 μM CoCl2 buffer at pH 7.2 and 37°C overnight. The PCR products were separated on 6.5% polyacrylamide 8 M urea sequencing gels and transferred to a Hybond-N+ nylon membrane (Amersham, Aylesbury, UK). They were hybridized for at least 2 hours at 42°C with the elongated primers, covalently labelled with peroxidase (ECL kit, Amersham). The membranes were washed once in 3 x SSC/0.1% SDS at 39-42°C for ~20 minutes and then twice for at least 15 minutes in 0.2 x SSC at 39-42°C. After washing, the membranes were bathed in a detection reagent containing H2O2, luminol and an enhancer (ECL kit, Amersham) for 1 minute at room temperature and the signals were detected on AGFA Cronex 5 film. We used the SPSS 9.0 software for Windows (SPSS Inc., 444 N. Michigan Avenue, Chicago, IL, 60611, USA) in this survival analysis.

Results

Loss of heterozygosity at the FHIT locus in tumour samples. The microsatellite polymorphism in the FHIT gene was analysed by screening, 397 solid human tumours using four intragenic and two adjacent markers. Of the 397 tumours analysed, 332 were informative for the markers. Information on marker location and LOH in the tumour samples analysed are presented in Figure 1. For the intragenic markers, we detected LOH for at least one marker in 166 tumours (50%), of which 114 had LOH at all informative markers, an indication of a large deletion in the FHIT gene (Figure 2, Table 1). The remaining 52 tumours with LOH, had a discontinuous LOH pattern, with LOH and ROH (retention of heterozygosity), indicating breaks within the FHIT gene (Table 1). As presented in Figure 2, the LOH proportion was variable in tumours from different locations. The lowest frequency of LOH with at least one of the markers was
detected in thyroid tumours (30%) and the highest frequency of LOH in lung tumours (66%). Based on the LOH percentage for the 9 different locations (Figure 2), the tumours could be arranged in the following ascending order: thyroid, endometrium, sarcoma, gastric, colorectal, ovary, testis, renal and lung. For most of the tumour types analysed, 65-85% of samples that had LOH for at least one intragenic marker also had LOH for all informative intragenic markers. The exceptions to this were endometrial and thyroid tumours, where the LOH for all markers was only detected in 20% and 33% respectively, of the samples with LOH at one marker (Figure 2).

Figure 2 shows that the LOH at D3S1234 and D3S1300 in gastric tumours was relatively low compared to D3S2757 and D3S4260. In colorectal tumours the LOH frequency was rather low on all the markers, ranging from 25% on D3S1234 to 35% on D3S4260 and no convincing peaks of LOH were detected. In contrast, the lung tumours had a high percentage of LOH at all the markers, with two peaks at D3S1300 (65%), which was the highest detected LOH for an individual marker in this study, and D3S4260 (62%). The LOH was also elevated at D3S4260 in gastric, ovary, testis and renal cancers.

In the endometrium tumour samples, the frequency of LOH was quite different depending on the markers, with 8% and 15% at D3S1234 and D3S4260 respectively, 36% at D3S1300 and 40% LOH at the D3S4260 marker. The ovary tumours had a similar proportion of LOH at three of the four markers, the fourth marker D3S4260 exhibiting a higher percentage of LOH, at 63%. A relatively high frequency of LOH was found at all of the markers in the testis tumours, the lowest at D3S1234 (39%) and the highest at D3S2757 (54%). The renal tumours had two peaks at D3S2757 (53%) and D3S4260 (55%). None of the markers reached above 25% LOH in the thyroid tumours, while with one of the markers, D3S2757, there was no LOH detected at all. In the sarcomas there was a peak of 38% LOH at D3S2757 but no LOH at D3S4260. However, the sample size was low for these tumour locations, with only 10 informative thyroid samples and 12 informative sarcoma samples.

Analysis of breakpoint location in the FHT gene. Breakpoint is a term used to identify juxtaposed regions where one region has LOH and the other has ROH. All tumour types showed this discontinuous LOH pattern for the intragenic markers to some extent, ranging from 7-25% of the total informative samples analysed (Table I) and 12-80% of the samples with LOH (Table II). Based on this information, the most common breakpoint inside the gene was between markers D3S1234 and D3S1300 in tumours from all locations except ovary, thyroid and sarcoma (Table II, Figure 1). The majority of breakpoints inside FHT in the ovary tumours were between markers D3S1300 and D3S2757 (Table II, Figure 1). There were only two FHT breakpoints in the thyroid tumours, one between D3S1300 and D3S2757 and the other between D3S2757 and D3S4260 (Table II, Figure 1). Only one FHT breakpoint was detected in the sarcoma samples, which was between markers D3S1300 and D3S2757 (Table II, Figure 1). For those samples with LOH at all intragenic markers, we made an attempt to locate breakpoints using two exogenous adjacent markers. These markers, D3S1313 and D3S1600, are located telomeric and centromeric, respectively, to the FHT gene (Figure 1). Adding these two markers increased the proportion of LOH samples with detected breakpoints for all tumour locations except sarcoma and endometrium. However, for most tumour types, the proportion of breakpoints that we were unable to locate using
analysed markers was still relatively high (Table II). This indicates that a large percentage of breakpoints are located outside the chromosomal region analysed and are therefore possibly at a larger distance from the FHIT gene. Most of the located breakpoints in lung tumours were inside FHIT; only 6% were located outside the intragenic markers, all centromeric to FHIT (Table II, Figure 1). This was reversed in testis cancer where most of the breakpoints located were found outside the intragenic markers. Thus, using 6 markers, we could locate 25-100% (average 58%) of the breakpoints within or close to the FHIT gene in the analysed LOH tumour samples (Table II). In those tumour types where the informative sample size was low (endometrium, thyroid and sarcoma), the percentage of breakpoints located in or near the gene was more extreme, i.e. 25% in the sarcoma tumours and 100% in the thyroid samples (Table II).

Association between LOH at the FHIT locus in colorectal cancer and patients' survival. Colorectal tumour was the only tumour type in this investigation that had an acceptable number of samples with and without FHIT LOH, to perform a comparative survival analysis based on FHIT LOH. We did in fact have a relatively large number of informative lung tumours, but most of them had LOH at least one marker, or 42 out of 64 (Figure 2); thus only 22 informative lung tumours had ROH. We would therefore have needed a larger sample size to do a survival analysis for patients with lung.

Table I. The LOH pattern in different tumour types, using four intragenic markers.

<table>
<thead>
<tr>
<th>Location</th>
<th>LOH on all informative markers</th>
<th>Discontinuous LOH pattern</th>
<th>ROH on all informative markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric</td>
<td>9/32</td>
<td>28</td>
<td>13/32</td>
</tr>
<tr>
<td>Colorectal</td>
<td>26/90</td>
<td>29</td>
<td>14/90</td>
</tr>
<tr>
<td>Lung</td>
<td>28/64</td>
<td>44</td>
<td>14/64</td>
</tr>
<tr>
<td>Endometrium</td>
<td>1/16</td>
<td>6</td>
<td>4/16</td>
</tr>
<tr>
<td>Ovary</td>
<td>13/35</td>
<td>37</td>
<td>4/35</td>
</tr>
<tr>
<td>Testis</td>
<td>13/27</td>
<td>48</td>
<td>2/27</td>
</tr>
<tr>
<td>Renal</td>
<td>20/46</td>
<td>44</td>
<td>7/46</td>
</tr>
<tr>
<td>Thyroid</td>
<td>1/10</td>
<td>10</td>
<td>2/10</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>3/12</td>
<td>25</td>
<td>1/12</td>
</tr>
<tr>
<td>Total</td>
<td>114/332</td>
<td>34</td>
<td>52/332</td>
</tr>
</tbody>
</table>
cancer. The same was evident for renal tumours and the sample size was too low for the rest of the tumour types.

The survival curves for colorectal cancer do seem to separate (Figure 3), indicating an increased mortality of colorectal cancer patients if they have FHIT deletions. The association was, however, not significant as tested with a log-rank test ($p = 0.12$). The follow-up time was 3 years. There were 40 cases of colorectal cancer patients with FHIT LOH; 55% were still alive after 3 years, and the mean survival time was 2.04 years (standard error = 0.18). There were 50 patients without FHIT LOH; 68% of them were still alive after 3 years and the mean survival time was 2.5 years (standard error = 0.12). We did not perform a multivariate analysis since the separation of the survival curves was not significant.

Discussion

The FHIT gene is fragile in several tumour types and FHIT knockout mice models support the idea of a haplo-insufficient mechanism for FHIT promoting tumour growth (37). Therefore analysis of intragenic markers is useful in screening for abnormalities of FHIT in tumour cells. We analysed LOH at 4 FHIT intragenic markers in 332 informative tumours from 9 different locations. The LOH detected with at least one microsatellite marker ranged from 30-66% depending on the location, being highest in lung tumours and lowest in thyroid tumours. These results clearly indicate that deletions within the FHIT gene are frequent events in cancer development. Using the intragenic markers, we were able to locate breakpoints in samples of all tumour types with LOH at the FHIT locus, ranging from 12-80%. With two exogenous markers adjacent to the FHIT gene, additional breakpoints could be located in the vicinity of the FHIT gene in up to 41% of the tumours. We analysed the association between LOH at the FHIT locus in colorectal cancer and patient survival. There seemed to be a trend towards a higher mortality among patients with FHIT LOH versus those with FHIT ROH.

It has been proposed that FHIT alterations are more common in tissues that are exposed to carcinogens from the environment (11). Therefore the LOH frequency in gastric (41%) and colorectal (44%) tumours was surprisingly low compared to, for example, ovary (49%) and testis (56%) tumours. However, the high frequency of LOH in ovary and testis tumours could be targeting a different 3p locus. In support of that concept was the relatively high proportion of LOH at all markers in ovary and testis tumours compared to colorectal and gastric tumours. There was a high frequency of LOH at all markers in lung tumours, and LOH at at least one marker was detected in 66% of lung cancer samples. We also observed a high frequency of FHIT LOH in renal tumours, or 59% at at least one informative intragenic marker, while
LOH at individual markers was 36-50%. The LOH frequency for the endometrial, thyroid and sarcoma cancers was approximately 30%, but the results for these locations were based on a small number of informative samples. Of those cases with at least one of the markers inside FHIT deleted, 65-85% had LOH for all informative markers inside the gene, suggesting large deletions. The exceptions were endometrium and thyroid tumours with 20% and 33% LOH for all informative intragenic markers, respectively, meaning the pattern of discontinuous LOH is more common in these tumours. The proportion of LOH at the FHIT locus in the colorectal, endometrial, gastric, lung and renal tumours analysed in this investigation was similar to previously reported data (14, 21, 23, 43-47).

We were able to identify discontinuous LOH in some of the tumour samples analysed, suggesting that there are breakpoints in the FHIT gene. But LOH at all informative intragenic markers was a more common pattern in most of the tumour types investigated, except endometrium and thyroid cancers. The pattern of LOH at all intragenic markers was in most cases 2-3 times more common than the breakpoint pattern, but in testis tumours it was about 7 times more common, indicating that a large region is commonly deleted in testis cancer.

In the majority of tumour types the most frequently occurring breakpoints were in the noncoding region of FHIT or between D3S1300 and D3S1234, both located in intron 5. We detected breakpoints, in most of the tumour types, between D3S1300 and D3S2757. These markers flank exon 5 implying that the exon is missing. Exon 5 contains the AUG initiation codon for the synthesis of the FHIT protein (1). A large part of the tumours analysed had all investigated loci inside the gene deleted. In those tumours we made an attempt to locate breakpoints by using the two markers flanking the gene. If there was ROH of either one of the markers we concluded that there was a breakpoint since all markers inside the gene had LOH. In some cases there were breakpoints on both sides of FHIT while in other cases we found only one of the loci outside the gene preserved, thus only one breakpoint located. Six to forty-one percent of the detected breakpoints were located outside FHIT in all tumour types, except sarcoma and endometrium. However, it must be emphasised that for most tumour types, there was still a relatively high percentage of breakpoints that could not be located. Thus, in many of the samples there were breakpoints outside the analysed chromosomal region. It cannot be excluded that, at least in some tumours, a complete loss of the whole chromosomal arm occurred. The majority of breakpoints that we could locate outside FHIT were centromeric (between D3S4260 and D3S1600) or nearly three times as many as telomeric (between D3S1234 and D3S1313). A possible explanation for this could be that D3S1600 is located at a greater distance from FHIT than D3S1313. In lung tumours there were only 3 breakpoints located, outside
FHIT, in 28 cases (6% of the detected breakpoints in lung cancer), all between D3S4260 and D3S1600, or telomeric. Wistuba et al. (48) also found breakpoints at FHIT in a more extensive study on lung cancer and reported an increase of frequency and size of deletions on 3p with increasing severity of histological change. The juxtaposed regions of LOH and ROH that we call breakpoints could occur as a cause of physical deletion and/or by mitotic recombination. It has been noted in a previous investigation that breakpoints or discontinuous LOH were present in several human tumours at a variety of chromosomal loci, and it was hypothesised that discontinuous LOH was a "signature" mutational pattern for oxidative damage which is widespread in human cancer (49).

We have also shown in this study that FHIT deletions seem to be associated with poor survival in colorectal cancer. Although not statistically significant, the survival curves showed an obvious trend towards separation with respect to FHIT LOH versus retention of heterozygosity, i.e. patients with FHIT LOH have an increased risk of dying. In other tumour types, such as breast, lung and stomach cancers, loss of FHIT is an indicator of poor prognosis (8, 50, 51).

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References


