MUTATION ANALYSIS OF THE *FHL2* GENE IN HUMAN PROSTATE AND MAMMAE CANCERS

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Are Bjarke Teigan *Marius Molund*

KULL 99

Veiledere:

Prof. Dr. Ugo Moens, UiTø, IMB, avd for biokjemi

Dr. Marijke Van Ghelue, UNN, avd for med. genetikk

TROMSØ

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RESYMÉ

Formålet med oppgaven var å undersøke om det finnes mutasjoner i *fhl2* genet i prostata tumorvev og/eller mammae tumorvev. Dette har aldri blitt gjort før og er høyst aktuelt. *Fhl2* genet vies stadig mer oppmerksomhet i cancer forskning.

Vi gjorde derfor en eksperimentell studie ved medisinsk genetisk avdeling. Vi utvant og isolerte DNA fra ferskt og parafin-innstøpt vev, fant fram til optimale annealing-temperaturer, renset og polymeriserte dette DNA og leverte prøver til sekvensering. Deretter ble resultatene analysert og vi gjorde et funn.

En mis-sense mutasjon ble funnet i nukleotid 154 i ekson 4 i 11 av 14 ca prostata vev, ingen funn i ca mammae vev. Den aktuelle mutasjonen innebærer endret aminosyresekvens i FHL2. For å få vite mer om viktigheten av dette funnet bør det forskes videre på effekten mutasjonen har på FHL2s aktivitet, og om mutasjonen kan forklare en rolle for FHL2 i onkogenese.

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INTRODUCTION

Cancer is one of the major causes of death in humans. In the year 2000, malignant tumors were responsible for 12 per cent of the nearly 56 million deaths worldwide from all causes. In many countries, more than a quarter of deaths are attributable to cancer. In 2000, 5.3 million men and 4.7 million women developed a malignant tumor and altogether 6.2 million died from the disease. Lung cancer is the most common cancer worldwide, accounting for 1.2 million new cases annually; followed by cancer of the breast, just over 1 million cases; colorectal, 940,000; stomach, 870,000; liver, 560,000; cervical, 470,000; oesophageal, 410,000; head and neck, 390,000; bladder, 330,000; malignant non-Hodgkin lymphomas, 290,000; leukemia, 250,000; prostate and testicular, 250,000; pancreatic, 216,000; ovarian, 190,000; kidney, 190,000; endometrial, 188,000; nervous system, 175,000; melanoma, 133,000; thyroid, 123,000; pharynx, 65,000; and Hodgkin disease, 62,000 cases. On average 1 in 16 women develop breast cancer. It is estimated that there will be 15 million new cases every year by 2020 [1].

An emerging picture in understanding the molecular mechanisms underlying cancer is the aberrant contribution of signal transduction pathways and transcription factors in tumorogenesis. In accordance with this, the spectrum of mutations in different genes encoding proteins that are components of signalling pathways or that function as transcription factors is accumulating [for recent reviews see 2,3]. Mutations in genes encoding transcription factors are also found in prostate cancer and breast cancer. The androgen receptor (AR) functions both as a signal transduction molecule and as a transcription factor. Mutations in the gene encoding the AR have been reported in prostate cancer and the AR is a target for endocrine therapy of prostatic carcinoma [4]. The breast cancer susceptibility gene 1 (*BRCA1*) encodes the BRCA1 protein, which plays a role in transcription regulation. Mutations in the *BRCA1* gene have been found in approximately 50% of hereditary breast cancers and in nearly all familial cases with a history of both ovarian and breast cancer [5].

The FHL2 transcription factor is a member of the LIM domain proteins. The LIM domain is a cysteine-rich motif with the consensus sequence $CX_2CX_{16-23}HCX_2CX_2CX_{16-21}CX$ (with C the amino acid cysteine, H the amino acid histidine, and X any amino acid). This domain mediates protein-protein interactions [6]. As expected for proteins with LIM domain structures, FHL2 is able to interact *in vivo* with numerous cellular proteins, including transmembrane receptors (e.g. integrins), cytosolic proteins having structural or other properties (e.g. insulin-like growth factor binding protein, presenilin-2), nuclear shuttling proteins (e.g. β-catenin), and transcription factors (e.g. AR, BRCA1, cAMP-response element binding proteins CREB and CREM, WT1 tumor suppressor, promyelytic leukaemia zinc finger protein 5, corepressor NcoR, myocyte nuclear factor, activator protein-1) [reviewed in 7]. FHL2 stimulates the transcriptional activity of AR, while the interaction between BRCA1 and FHL2 enhances the transcriptional properties of FHL2 [5,8]. While endogenous FHL2 resides to the cell membrane in the secretory epithelium of normal human prostate tissue, a significant increase in nuclear localization was observed in prostate cancer tissue samples obtained from different patients. In fact, increasing nuclear localization of FHL2 correlates with progression to a highly malignant phenotype of prostate carcinoma cells [9]. The *in vivo* association of FHL2 with AR or BRCA1, and the aberrant subcellular distribution of FHL2 in prostate cancer tissue may suggest a role for this protein in tumorogenesis.

We hypothesized that mutations in the *fhl2* gene may alter the subcellular localization of FHL2 or may affect the transcriptional activity of *fhl2* itself or of its partners AR and BRCA1. These changes in normal FHL2 functions could therefore contribute to the malignant phenotype of the affected cell. This assumption prompted us to compare the coding sequences of the wild-type *fhl2* gene with the sequences in human prostate cancer and breast cancer samples. The human *fhl2* gene is located on chromosome 2 and spans 38,400 base-pairs (bp). The gene consists of 8 exons and is transcribed into a 1874 bases long mRNA. The coding sequence in the mRNA encompasses 840 bases, which results in a 280 amino acid long FHL2 protein after translation.

By polymerase chain reaction (PCR) on total cellular DNA obtained from mammae cancers and prostate cancers, all eight exons were amplified. The sequences of all exons in these samples were determined and compared with the sequences of normal tissue available in the sequence database. Our results show a missense mutation in nucleotide no 154 in exon 4,

exclusively in the tissue from ca. prostate, in 11 of our 14 prostate tissues. This point mutation results in the substitution of lysine into glutatmate.

MATERIALS AND METHODS

Fresh tissue

Nine samples from cancer mammae and 1 sample from normal prostate were obtained from the Department of Pathology, University Hospital Tromsø.

Paraffine-embedded tissue

Fourteen samples were obtained from the Department of Pathology, University Hospital, Tromsø

Isolation of DNA

DNA was isolated using BioRobot E21 and E21 DNA blood 350 µl kit, Qiagen according to the manufacturer's protocol (Qiagen Gmbh, Hilden Germany).

PCR

Table1: Primers used in PCR and sequencing reaction

The PCR mix used Jump Start AccuTaq LADNA Polymerase Mix purchased from SIGMA, St. Louis, Missouri 63103, USA

PCR products were analyzed by agarose gel electrophoresis.

Sequencing PCR product:

PCR products were sequenced using BigDye®Terminator v1.1 cycle sequencing kit and BigDye®Terminator v 1.1 v 3.15x sequencing buffer purchased from Applied biosystems, Foster City, California 94404, USA. Sequencing reactions were analyzed on an ABI 377 Prism Sequencer (Peikm-Elmer).

Analyses of the sequences

Sequences were analysed using SeqScape v.2.1 from Applied Biosystems

RESULTS

Table 2: PCR conditions, Fresh material

Table 3: PCR conditions, DNA extracted from paraffine embedded material

Optimalization of the PCR conditions

The *fhl2* gene consists of 8 exons interrupted by 7 introns, and the entire gene spans 38,400 bp. Prior to determining the sequence of the gene, we amplified the DNA encompassing the gene. The amplified DNA was then used for sequencing. Amplifying 38,400 bp in vitro by DNA polymerase is very difficult. Therefore, a specific primer set for each exon was designed that allowed the amplification of the individual exons. The individual amplified exon regions could thereafter be sequenced. First, a temperature gradient PCR was run to optimize the annealing temperature for each individual primer set. The temperatures that gave optimal amplification of the exons are summarized in Table 2 and 3. Minor differences were found for some of the same primer sets when DNA extracted from fresh or paraffin-embedded tumour tissue was used (e.g. compare annealing temperatures for the primers exons 3, exon 6, and exon 8, respectively). The optimal annealing temperatures were subsequently used in all PCR.

Mutation analyses of *fhl2* gene in prostate cancer and cancer mammae

Nine fresh samples from cancer mammae and fourteen samples of paraffin-embedded prostate cancer tissue were available. In addition, one control sample of normal prostate tissue was analyzed. PCR amplification products were obtained for all exons of all samples. Since the start codon for the FHL2 protein resides in exon 3, we concentrated on sequencing exons 3 to 8. No mutations were found in any of the breast cancer samples. However, eleven out of 14 prostate cancer samples displayed a missense mutation (AAG→GAG) compared to the

sequence of non-diseased tissues, changing a lysine residue into a glutamate. A silent mutation (CCC→CCT, proline) was found in exon 8 in some of the samples.

DISCUSSION

Breast cancer and prostate cancer are very common among the human population. Mutations in the genes encoding the androgen receptor (AR) and the BRCA1 protein have been found in prostate cancer and cancer mammae, respectively. Recently, the FHL2 protein was shown to interact with both the AR and BRCA1 and this association affected the activity of interacting partners. Therefore, it can be assumed that disturbance in normal FHL2 function may affect the properties of AR and BRCA1, and this may contribute to malignancy. This urged us to investigate whether mutations in the *fhl2* gene could be found in these cancers. In this study, our preliminary results show a missense mutation in nucleotide 154 in exon 4 of the *fhl2* gene. This mutation was exclusively present in prostate cancer tissue and not in cancer mammae. Eleven out of 14 (78.5%) of the prostate cancer samples possessed this mutation. The mutation changed the basic amino acid lysine (K) at position 52 in the FHL2 protein into the acidic amino acid glutamate (E). The mutation was located at the end of the sequenced DNA fragment, in a region where interpretation of the sequence data should be interpreted with care. To confirm the presence of this mutation, additional sequencing should be performed with primers closer to the region of interest because the sequence determined in the proximity of the primer are less error-proned than sequences further away from the primer. Alternatively, the complementary strand of the DNA can be sequenced to verify the presence of the mutation. Due to limited time of the project, this could not be done.

To our best knowledge, this is the first study that has screened malignant tissues for mutations in the *fhl2* gene and the first study to report a putative mutation in this gene, which seems to be cancer tissue-specific. Previous studies have compared the expression levels of FHL2 in various cancer mammae and prostatic carcinoma cell lines with normal tissue. The presence of FHL2 mRNA was monitored in three prostatic carcinoma cell lines by reverse transcriptase PCR (RT-PCR). Transcripts were found in the DU-145 and PC-3 cell lines, but not in LNCaP cells. Moreover, FHL2 mRNA could be detected in two different primary stromal cell cultures derived from normal prostate tissue. However, the FHL2 mRNA levels were not quantified,

making the interpretation and biological relevance of these findings difficult to interpret [4]. Another group compared FHL2 transcription levels in different breast cancer cell lines by a semi-quantitative RT-PCR method. No transcripts were detected in BT-474 cells, while FHL2 mRNA was present in T47D, MCF-7, ZR-75-1, and MFM 233 breast cancer cell lines. The lowest levels were found in ZR-75-1, while 2-, 4-, or 8-fold higher levels were measured in MFM223, MCF-7, and T47D cells, respectively [10]. We have not examined our clinical samples for the expression levels of the *fhl2* gene.

It remains to be established whether the mutation described in this study affects the normal biological function of FHL2 and whether this mutated FHL2 protein is involved in prostate cancer. Lysine at position 52 is conserved in mouse, rat and human FHL2, and also in the closely related FHL1 of different species and ACTS proteins [11]. The evolutionary conservation underscores the importance of this amino acid residue in the function or/and structure of FHL2. However, the substitution of a lysine into a glutamate is potentially interesting. Lysine is a positively charged amino acid, while glutamate is negatively charged. This inversion of charge may have profound effects on the conformation of the protein. This may change the properties of the protein or may alter the interaction with other proteins. Indeed the region containing K52 is part of one of the regions involved in the interaction with AR [8], and is also part of the transactivator domain of FHL2 [11]. Moreover, lysine residues are often target sites for acetylation. Acetylation of a protein can affect its subcellular localization, its interaction with other proteins, its stability [reviewed in 12]. A previous study has shown a more profound nuclear localization of FHL2 in prostate cancer tissue and increasing nuclear localization of FHL2 correlated with progression to a highly malignant phenotype of prostate carcinoma cells [9]. Immunohistological studies of the prostate cancer samples with the described mutation could shed light on this. Alternatively, studies with fusion proteins of FHL2 and green fluorescence protein (GFP) can be performed. The GFP allows easily microscopic detection due to its fluorescence. The subcellular localization of wild-type FHL2-GPF can be compared with that of FHL2K52E-FHL2. We have also a test system that allows us to compare the transcriptional activity of wild-type FHL2 and FHL2K52E.

We cannot exclude that other mutations in the *fhl2* gene can affect the expression of this gene. Mutations in the promoter region of this gene may increase or decrease the transcription level of the gene. Alternatively, mutations in the introns may affect the splicing of the mRNA, thus

resulting in other splicing-variants of FHL2 mRNA, which generate other isoforms of the protein. These isoforms may exhibit altered functions. Our primers were designed that also part of the introns were amplified during the PCR. We were unable to detect any mutations in these regions. Finally, mutations in the 3´untranslated region of the messenger may result in stabilization or destabilization of FHL2 mRNA, thereby affecting the levels of this protein.

It is of great interest to examine this gene further, especially exon 4. As a first step, a larger number of cancer samples should be investigated to confirm our findings and to increase the correlation of this mutation with prostate cancers. In addition, we should examine different tissues of the patients to establish whether this mutation is present in the DNA of all cells. This may indicate whether the mutation is inherited or induced during life. We should also find out whether both alleles are affected in the tumour tissue. Such data may provide information on the recessive or dominant nature of the mutant. Furthermore, biochemical and biological studies should be performed to understand the consequences of this mutation on the function of the protein. Knock out mice with mutated *fhl2* gene or transgenic mice overexpressing mutated FHL2 in e.g. a prostate tissue-specific way can be generated to explore the potential oncogenic role of FHL2.

In conclusion, our preliminary results show for the first time a prostate cancer-specific mutation in the transcription factor FHL2. The biological consequences of this mutation are unknown and it remains to be established whether the *fhl2* gene is a genuine target gene in malignancy of the prostate tissue. If so, diagnostic screening may help to identify patients predisposed to this type of cancer and an early follow-up of these patients may allow immediate identification of the development of prostatic cancer and increase the survival changes of affected men.

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TABLES AND FIGURES

Table1: Primers used in PCR and sequencing reaction

Primer:	Sequence:	Supplier:
fhl2 Exon 1F	5`- TACTGTGCTCCCAAGACCCG- 3`	Sigma Genosys
fhl2 Exon 1bR	5`- CCCACAGCTCTGCTCT- 3`	Sigma Genosys
fhl2 Exon 2 F	5`- TTCGCTTCCTCAAGATGACC- 3`	Eurogentec, 4102 Seraing-Belgium

fhl2 Exon 2 R	5`- CAGTAGCTAAAACTGAAATCCAGAA-3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 3 F	5`- TGTGGAGCCCTACTTATTTTC- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 3 R	5`- TGAAACCCCATCTCTACTAA- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 4 F	5`- CCCCTTTCAGAGAGTGCTTAA- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 4 R	5`- AGAGAAACCCGTGTTTCCTA- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon5 F	5`- TCCAAGCCCTCCTGTCCT- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 5 R	5`- TGCACAAGGCTTGAAGCTAGA- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 6 F	5`- TCCACACCCCTCTAACCATAA- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 6 R	5`- AAACCAGCCAGAACGTGCA- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 7 F	5`- TGAAGATGTCTGCCAAGCA- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 7 R	5`- TTGTTCAAGCTTCCAATCGC- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 8 F	5`- TCCCAGTTGCCTGATTCTAA- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 8 R	5`- AGTGCCTCGACCTGTAATCAT- 3`	Eurogentec, 4102 Seraing-Belgium
fhl2 Exon 8cR	5`- GATTGCCTGGGTGAGAAAGA- 3`	Sigma Genosys

Table 2: PCR conditions , Fresh material

	Stage 1	Stage 2: 30 cycles			Stage 3	Stage 4
Primers	Denaturation	Denaturation	Annealing	Extention	Extention	Hold

Exon 1F Exon 1R	95°C 4 min	95°C 30 sek	63,4°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 2 F Exon 2 R	95°C 4 min	95°C 30 sec	67,6 °C 30 sec	72°C 3 min	72°C 7 min	4° C
Exon 3 F Exon 3 R	95°C 4 min	95°C 30 sec	61,3°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 4 F Exon 4R	95°C 4 min	95°C 30 sec	63,4°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 5 F Exon 5 R	95°C 4 min	95°C 30 sec	69,8°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 6 F Exon 6 R	95°C 4 min	95°C 30 sec	63,4°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 7 F Exon 7 R	95°C 4 min	95°C 30 sec	67,6°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 8 F Exon 8 R	95°C 4 min	95°C 30 sec	61,4°C 30 sec	72°C 3 min	72°C 7 min	4°C

Table 3: PCR conditions, DNA extracted from paraffine embedded material

	Stage 1	Stage 2: 30 cycles			Stage 3	Stage 4
Primers	Denaturation	Denaturation	Annealing	Extention	Extention	Hold
Exon 1F Exon 1R	95°C 4 min	95°C 30 sek	63,4°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 2 F Exon 2 R	95°C 4 min	95°C 30 sec	67,6 °C 30 sec	72°C 3 min	72°C 7 min	4° C
Exon 3 F Exon 3 R	95°C 4 min	95°C 30 sec	57,8°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 4 F Exon 4R	95°C 4 min	95°C 30 sec	63,4°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 5 F Exon 5 R	95°C 4 min	95°C 30 sec	69,8°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 6 F Exon 6 R	95°C 4 min	95°C 30 sec	65,0°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 7 F Exon 7 R	95°C 4 min	95°C 30 sec	67,6°C 30 sec	72°C 3 min	72°C 7 min	4°C
Exon 8 F Exon 8 R	95°C 4 min	95°C 30 sec	58,0°C 30 sec	72°C 3 min	72°C 7 min	4°C