**APC I1307K mutations and forkhead box gene (FOXO1A): another piece of an interesting correlation**

Marco Agostini¹,², Chiara Bedin¹,², Salvatore Pucciarelli¹, Mariavittoria Enzo¹,², Marta Briarava¹, Roberta Seraglia³, Eugenio Ragazzi³, Pietro Traldi³, Laura Molin⁴, Emanuele Damiano Urso¹, Isabella Mammi⁶, Alessandra Viel⁷, Mario Lise¹, Ennio Tasciotti², Alessandra Biasiolo⁸, Patrizia Pontisso⁸, Donato Nitti¹

¹Department of Surgical, Oncological and Gastroenterological Sciences, 2nd Surgical Clinic, University of Padua, Padua - Italy
²The Department of Nanomedicine, The Methodist Hospital Research Institute, Houston, TX - USA
³Pediatric Research Institute - Città della Speranza, Padua - Italy
⁴CNR-ISTM, Padua - Italy
⁵Department of Pharmacology and Anesthesiology, University of Padua, Padua - Italy
⁶ULSS 13 Dolo, Venice - Italy
⁷Division of Experimental Oncology, Centro Riferimento Oncologico, IRCCS, Aviano - Italy
⁸Fifth Medical Clinic, Department of Clinical Experimental Medicine, University of Padua, Padua - Italy

These authors contributed equally to this article.

**ABSTRACT**

*Purpose:* Germline nonsense and frameshift mutations in the adenomatous polyposis coli (APC) gene are found in approximately 90% of individuals affected by familial adenomatous polyposis (FAP) and a genotype-phenotype relationship has been observed. Missense mutations have also been found in a few cases, even if their role in FAP is still unknown. An association between a missense mutation, APC I1307K, and the risk of sporadic colorectal cancer (CRC) has been reported. In order to improve the knowledge about the genetic effect of APC I1307K on the phenotype, we tried a new approach using matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS).

*Experimental design:* An APC mutation (I1307K) was found in an index case of a non-Jewish woman and her son with attenuated familial adenomatous polyposis (A-FAP) and no family history of cancer. In order to evaluate whether the presence and abundance of the ionic species are related to the presence of cancer or the presence of mutation, comparative analyses of 11 healthy clean-colon subjects, 59 patients with CRC (stage II n=19, stage III n=23, stage IV n=17) without polyps, and 9 FAP patients, carriers of a nonsense mutation in the APC gene, were evaluated.

*Results:* Comparative analysis of serum protein profiles of the index patient and her healthy son, FAP and sporadic CRC patients, and subjects with preneoplastic lesions showed a characteristic abundance of ionic species at m/z 905, which was not present in healthy controls. Two peptides were identified from MALDI/MS/MS spectra of m/z 905 belonging to the kininogen-1 precursor and the human forkhead box protein 01A (FOXO1A). FOXO1A was present in only two subjects carrying I1307K, but not in other patients.

*Conclusions:* Our findings seem to suggest a relationship between m/z 905, FOXO1A and the development and growth of colorectal cancer. FOXO1A fragment determination in serum with MALDI/MS might be a promising approach for early detection of colon carcinoma or for the development of targeted therapies.

**Key words:** FOXO1A, Colorectal cancer, APC I1307K

Received: June 10, 2011; Accepted: August 30, 2011

**INTRODUCTION**

Familial adenomatous polyposis (FAP) has autosomal dominant inheritance caused by mutations in the adenomatous polyposis coli (APC) tumor suppressor gene located on chromosome 5q. Pathogenetic germline mutations in the APC gene are nonsense and frameshift in about 90% of affected individuals (1) and a genotype-phenotype relationship has been observed (2). Missense mutations have also been found in a few cases, even if their role in FAP is still unknown. In a mouse model, different APC mutations confer different degrees of susceptibility to tumorigenesis (3). APC I1307K is a missense mutation involving a T>A...
transversion that should not have detectable effects on APC function and might, therefore, be classified as a silent polymorphism. However, an association between APC I1307K and the risk of sporadic colorectal cancer (CRC) has been reported (4, 5). In Ashkenazi Jews, the frequency of heterozygosity of APC I1307K ranges from 5% to 7% and from 8% to 15% in healthy and CRC subjects, respectively. The relative risk of developing CRC for individuals with this allele has been calculated to be 1.5-2.2 (4, 6) and seems to be associated exclusively with Ashkenazi ethnicity. T>A transversion creates a stretch of 8 adenines, which is thought to lead to errors in the replication process. Such errors cause a predisposition towards somatic single-nucleotide insertions or deletions inside or in the immediate vicinity of the (A)_8 stretch, leading to frameshifts. Tumorigenesis is apparently initiated when the wild-type allele is somatically mutated in an APC I1307K carrier (4, 7, 8).

In order to improve the knowledge about the genetic effect of APC I1307K on the phenotype and its correlation with serum protein profile, we tried a new approach using matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS). With this approach we found in a previous investigation that MALDI/MS was able to detect low-molecular-weight (LMW) serum proteins which are diagnostic for CRC (9) and in this study the presence of degradation products of the forkhead box O1A (FOXO1A) seems to indicate an early event associated with tumor growth.

FOXO1A is a member of the FOXO family of forkhead/winged helix transcription factors (11). FOXO transcription factors serve as signaling integration points. FOXO factors are phosphorylated (pFOXO1A) by protein kinase Akt in response to cellular stimulation by growth factors or insulin, and this event relocalizes FOXO proteins from the nucleus to the cytoplasm. FOXO factors are emerging as key tumor suppressors, as shown by the following examples: 1) FOXO factors mediate the cytostatic response of TGF-beta, a key cyotostatic pathway in CRC, by binding to Smads (12); 2) FOXO is essential for the TGF-beta-mediated upregulation of the CDK inhibitors P21WAF-1 and P15INK4A (12); 3) recent evidence has shown that β-catenin binds to FOXO and that this binding leads to enhanced FOXO transcriptional activity, suggesting that interaction of FOXO and β-catenin competes with binding of β-catenin with T-cell factor (TCF) (13); 4) expression of pFOXO1A is an early event of intestinal and gastric tumorigenesis (14); 5) pFOXO1A is correlated with 5 tumor suppressor genes (APC, SMAD4, CDKN2A, CD82, and PTEN) as well as with BCL2 (14); 6) induction of caveolin-1 by APC is mediated by FOXO1A and c-MYC (15).

Additional tumor-suppressive functions of FOXO include its ability to induce differentiation and to preserve genomic integrity. These combined properties make FOXO a critical determinant of the life span and of the proliferation and growth of CRC in mouse models (16). In normal cells, phosphorylation by mitogenic signals use FOXO factors to transiently relocalize from the nucleus to the cytoplasm, where the FOXO factors can be degraded via the ubiquitin-proteasome pathway. Although the major mechanism of FOXO regulation is mediated through changes in subcellular localization, altering FOXO protein levels can also have dramatic effects in the organism. In contrast to changes in subcellular localization, which are rapidly reversible, changes in FOXO protein levels are more permanent, and may have a profound impact on FOXO functions (17, 18).

MATERIALS AND METHODS

The index case of an APC I1307K carrier

A serum sample of a 53-year-old non-Jewish woman (indicated here as PD-4) with an APC I1307K mutation (first case of APC I1307K in a non-Jewish Italian family), referred to us in 2004 for polyposis of the colon with no family history of cancer, was used for the analysis.

She was diagnosed at the age of 45 years with a papillary thyroid carcinoma. The proband underwent colonoscopy because of increasingly frequent bloody stools, weight loss, and the detection of multiple polyps (>50). She underwent a subtotal colectomy. At histopathological examination, 80 tubular adenomas with moderate dysplasia were found. Based on the number of polyps found at histopathology and a careful workup, a diagnosis of attenuated FAP (A-FAP) without extracolonic manifestations was made. Both the asymptomatic unaffected 18-year-old daughter (named here PD-33) and 20-year-old son (PD-34) underwent colonoscopy without evidence of polyps in 2004. In 2010, PD34 showed the presence of a few polyps.

On the DNA extracted from the blood of the proband and her children, a mutation analysis for the APC and MYH genes was performed using polymerase chain reaction (PCR), direct sequencing, and multiplex ligation-dependent probe amplification (MLPA).

Serum samples and MALDI and MALDI/MS/MS measurements

Ten milliliters of peripheral blood was drawn into a blood collection tube (without EDTA additive) before physical examination (colonoscopy) and biopsy. Blood samples were left at room temperature for molecular assays and all were transferred to the study laboratory within 6 hours of collection for processing. Serum samples were obtained by centrifugation of 10 mL of peripheral blood at 1800g for 15 minutes. The samples were carefully collected from the upper portion of the supernatant and stored in aliquots at –80°C.

The profiles of LMW serum proteins in the samples were obtained with MALDI/MS using a Voyager-DE PRO
Agostini et al

Statistical analysis

FOXO1A values were log-transformed (ln-FOXO) in order to relax the assumption of normal distribution. Analysis of variance (ANOVA) was used to compare the mean values of ln-FOXO expression among different tissue samples. Using the median value of FOXO as the cutoff, samples were classified into high-expression vs low-expression subgroups, and the Cochran-Armitage test was used to verify the hypothesis of a linear trend between FOXO and different tissue samples ordered according to the following sequence: normal, adenoma, stage I, stage II, stage III, stage IV.

All statistical analyses were performed using the STATA SE 11.0 software (StataCorp, College Station, TX, USA)

RESULTS

On the DNA extracted from the blood of the proband, PD-4, a missense I1307K in heterozygosis was found in APC, while no mutations were found in the MYH gene. The same mutation was found in the DNA extracted from normal colon mucosa. In the adenoma, loss of heterozygosity (LOH) with loss of the wild-type allele was found, but without the characteristic somatic shifts (ins/del A) of this pre-mutation. DNA was extracted from blood samples of the children of the proband and tested for APC I1307K, which was found in both.

The profiles of LMW serum proteins of samples from proband PD-4 and her children, PD-33 and PD-34, were then obtained with MALDI/MS. The spectra of LMW

FOXO1A expression analysis

In colorectal tumors used for LMW analysis and additional cases in which frozen tissue was available, FOXO1A gene expression analysis (Hs01054576_m1, TaqMan® Gene Expression Assays, Applied Biosystems) was performed using quantitative PCR. Following the TNM AJCC staging system, 66 cases were evaluated: n=20 adenomas, n=16 stage I, n=5 stage II, n=20 stage III, n=5 stage IV. The RNA required for the analysis was extracted from frozen tumor tissue samples using a commercial kit (Qiagen). To normalize the results, the housekeeping gene, hypoxanthine phosphoribosyltransferase 1 (human HPRT1, TaqMan® Endogenous Control, Applied Biosystems) was used.

Immuno dot blot binding assay for FOXO1A quantification

Serum samples were spotted in triplicate onto a nitrocellulose membrane (Amersham Biosciences, UK) at 2 μL per spot and then allowed to dry. To block nonspecific sites, the membranes were incubated with 5% BSA containing TBS-Tween 0.05% (TBS-T) for 1 hour at room temperature in gentle soaking. After 3 TBS-T 0.05% washes (5 minutes each), the membranes were incubated with affinity-purified rabbit anti-FOXO1A antibody (Bethyl Laboratories, Montgomery, TX, USA) at 1:1000 dilution in BSA 0.1% - TBS-T 0.05% for 30 minutes at room temperature, washed 3 times, and then probed for 30 minutes with horseradish-peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO, USA) at 1:5000 dilution. After 3 final washes with TBS-T 0.05%, membranes were developed using an enhanced chemiluminescence substrate (ECL Advance, Amersham Biosciences, UK) and the intensity of the dot area was detected by the VersaDoc imaging system (BioRad, Milan, Italy). To estimate the concentration of FOXO1A in individual samples, a calibration curve obtained from serial dilutions of FOXO1A peptide (Bethyl Laboratories) (range 80 ng/mL-1.2 ng/mL) was included in each assay.

Fig. 1 - Ionic species (expressed as m/z) and their relative abundance found in serum of the three I1307K carriers, but not found in healthy controls at a high frequency (>10-20%).
protein species detected (measured as mass/charge ratio: m/z) and expressed as relative abundance, are reported in Figure 1. Differences were found between the three I1307K subjects; however, the LMW protein profile of the proband (PD-4) was very similar to that of her son PD-34, particularly with respect to the ionic species at m/z 905, 921, 1061, 1077 and 1897, whereas the LMW protein profile of the daughter, PD-33, showed a low abundance of the above-mentioned ionic species and a higher level of species at m/z 1865, 2005 and mostly 2021.

By comparing the serum spectra profile of the present family and healthy clean-colon subjects, the ionic species that were present in both groups were considered nonpathological and subsequently excluded. The result is shown in Figure 1, which reports the LMW species that might be linked to the family status, since they were not frequently found in healthy controls. It must be noted that the species at m/z 905 and 921, abundant in patient PD-4 and in her son, PD-34, were not present in the healthy control subjects with a frequency >10-20% and a relative abundance >3%.

A further comparison between the serum protein spectra of the index family and CRC patients showed that there were only 2 LMW species in common between the groups: m/z 905 and 1349 (9) (Fig. 2). The ionic species at m/z 905 appeared highly expressed in both patient PD-4 and her unaffected son, PD-34. Finally, the analysis of FAP patients with an APC nonsense mutation showed a high abundance of protein species at m/z 905 (10) (Tab. I). The species at m/z 1349 was found in PD-34 and CRC patients, but with low abundance, suggesting that it might not be specific. When the data analysis was carried out using only the LMW species found in the serum of both children, PD-33 and PD-34 (without cancer), and absent in their mother, PD-4 (with cancer), some species (at m/z 1865, 1934, 2005) were found to be in common with healthy subjects and absent in CRC patients.

However, in order to exclude that m/z 905 was a mass artefact, a group of 9 other subjects with adenomas was analyzed and in 22% of them, the same finding was present in relatively high abundance (Tab. I)

Since CRC patients and subjects at risk of CRC were characterized by the presence of the protein species at m/z 904-905 (9), a further investigation was undertaken by MALDI/MS/MS on the amino-acid sequence of this peptide, with the aim of identifying its origin.

The MALDI/MS/MS spectra of the species at m/z 904-905 obtained from serum samples of a CRC patient and of the proband and the children of both of them showed a similar pattern: an accurate analysis of the spectra suggests that for m/z 904-905 they are due to fragment ions of 2 different species.

The MS/MS spectra were compared with the Swiss-Prot.2006.02.21 database. Data analysis indicated that the first peptide is due to the sequence RPPGFSPF, found in the kininogen-1 precursor (human KNG1), which is an α-2-thiol proteinase inhibitor, and the second entry is due to the sequence PRKSSSSR, indicated as belonging to the human forkhead box protein 01A (forkhead in rhabdomyosarcoma, FOXO1A) (10).

In serum samples from a general population of CRC and FAP patients carrying a nonsense mutation, there was only human KNG1 peptide, while in two I1307K carrier subjects both human KNG1 and FOXO1A were present. Although, as previously described, the KNG1 peptide appears to be associated with the presence of CRC, in this study FOXO1A degradation products were present in the serum of 2 members of the index family with a risk of CRC, and of another subject with adenoma. These findings seem to indicate that the presence of degradation products of FOXO1A can be an early event associated
with tumor growth and transformation.

Therefore, our present identification of FOXO1A fragments in serum in preneoplastic lesions and in two I1307K carriers suggests that this homeostatic mechanism may become corrupted, favoring a more stable escape from the tumor suppressive functions of FOXO. However, the tumor suppressive functions have been confirmed in this study analyzing FOXO1A gene expression in a series of colorectal tumors used for LMW analysis and additional cases in which frozen tissue was available (Fig. 3).

Since FOXO's values were not normally distributed, a logarithmic transformation was performed. Compared to the levels of FOXO1A expression in carcinoma tissue, the levels in normal mucosa were significantly higher (p<0.05) and a trend towards an inverse association with pTMN stage seemed to be present: the expression level decreased with tumor progression (Tab. II, Fig. 3A). These results correlate well with those obtained with our proteomics approach, which found an association between the presence of fragments of FOXO1A and tumor growth, as shown also by immuno dot blot binding data (Fig. 3B).

**DISCUSSION**

Our results seem to indicate that the decrease in FOXO1A expression and the relatively high abundance of peptide fragments are an early and progressive event in colon cancer development. This finding seems to support the notion that FOXO1A is a tumor suppressor

**TABLE I - RELATIVE ABUNDANCE AND FREQUENCY OF OCCURRENCE OF m/z 905 SPECIES FOUND IN SERUM OF INDEX FAMILY MEMBERS AND SUBJECTS WITH PRENEOPLASTIC LESIONS, FAMILIAL ADENOMATOUS POLYPOSIS, COLORECTAL CANCER AND HEALTHY CLEAN-COLON SUBJECTS**

<table>
<thead>
<tr>
<th>Category</th>
<th>Disease</th>
<th>APC mutations</th>
<th>Mean relative abundance (%)</th>
<th>Frequency (%)</th>
<th>MS/MS analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-04</td>
<td>Polyposis</td>
<td>I1307K</td>
<td>100</td>
<td>-</td>
<td>Kininogen-1 precursor (human KNG1) Forkhead box protein 01A</td>
</tr>
<tr>
<td>PD-34</td>
<td>Healthy relative</td>
<td>I1307K</td>
<td>100</td>
<td>-</td>
<td>Kininogen-1 precursor (human KNG1) Forkhead box protein 01A</td>
</tr>
<tr>
<td>PD-33</td>
<td>Healthy relative</td>
<td>I1307K</td>
<td>9</td>
<td>-</td>
<td>Kininogen-1 precursor (human KNG1)</td>
</tr>
<tr>
<td>Preneoplastic lesion population (n=9)</td>
<td>Adenomas</td>
<td>wt</td>
<td>55</td>
<td>22</td>
<td>Not evaluated</td>
</tr>
<tr>
<td>FAP subjects (n=9)</td>
<td>Polyposis</td>
<td></td>
<td>60</td>
<td>44</td>
<td>Kininogen-1 precursor (human KNG1)</td>
</tr>
<tr>
<td>CRC population (n=59)</td>
<td>Colon-rectum cancer stage II n=19</td>
<td></td>
<td>37</td>
<td>60</td>
<td>Kininogen-1 precursor (human KNG1)</td>
</tr>
<tr>
<td>Control group (n=11)</td>
<td>Healthy clean colon</td>
<td></td>
<td>&lt;10-20</td>
<td>&lt;3</td>
<td>Not evaluated</td>
</tr>
</tbody>
</table>
APC I1307K mutations and forkhead box gene (FOXO1A): another piece of an interesting correlation

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Adenoma</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>13</td>
<td>13</td>
<td>5</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
<td>7</td>
<td>11</td>
<td>4</td>
<td>13</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>20</td>
<td>16</td>
<td>5</td>
<td>20</td>
<td>5</td>
<td>79</td>
</tr>
</tbody>
</table>

Exact 2-sided p value <0.0001

A gene that may be inactivated also through transcriptional downregulation.

Moreover, overexpression of the species at m/z 905 was present in a general population of CRC and FAP patients carrying a nonsense mutation, in two I1307K carriers of the index family, and in subjects with colorectal adenoma, while it was absent in one member of the index family and in healthy clean-colon controls (Tab. I).

These data seem to suggest a relationship between the overexpression of species at m/z 905 and the presence or risk of CRC and/or mutation in the APC gene. Moreover, the overexpression of other species (at m/z 1865, 1934, 2005) seems to be associated with the absence of cancer. In fact, the above species were overexpressed in the 2 members of the index family without cancer and in clean-colon healthy subjects, and absent in CRC patients.

Furthermore, the forkhead box genes may have an emerging role in colon cancer. Our results showed that the FOXO1A gene is upregulated in nontumor cells, and that a decrease in FOXO1A expression and the abundant presence of degradation products in serum samples are early and progressive events in colon cancer development. This finding supports the notion that FOXO1A is a tumor suppressor gene that is inactivated also through transcriptional downregulation. It seems that not all patients suffering from preneoplastic lesions have the same risk of progressing to cancer. The increased presence of degradation products could make FOXO1A a clear and distinctive indication of a progressive loss of opposition to carcinogenesis.

These preliminary data are interesting as a stimulus for further investigation. More subjects are required to confirm these preliminary results and the relationship between the expression of some species of LMW serum protein (i.e., m/z 904-905), the presence of germline APC mutations and/or the presence of CRC deserve more in-depth study. Moreover, identification of the overexpressed species is crucial in order to understand which proteins are involved and what is their role in carcinogenesis. From this point of view, the m/z 904-905 species and FOXO1A require further attention in patients with advanced CRC and in carriers of germline APC mutations at risk of cancer. We could speculate that the presence of species at m/z 904-905 in the proband and in 1 of her unaffected children may be linked to the susceptibility of developing CRC; however, it may also be a chance association. On the other hand, some species appear to be associated with healthy conditions, and from a clinical point of view, they may be as important as the markers of CRC.

The next steps will be to confirm these preliminary results in a larger cohort of CRC and FAP patients and extensive analysis of the FOXO gene will be necessary.

**Financial support:** This study was supported by Clinica Chirurgica II Tumor Tissue Biobank, grants from LILT (Lega Italiana per la Lotta Contro i Tumori), the Italian Ministry of Health (Programma Speciale ex. art.12), and AIRC (Associazione Italiana per la Ricerca sul Cancro).

**Conflict of interest statement:** We have no conflicts of interest to declare.

**Meeting Presentation:** The preliminary data of this study were displayed as a poster presentation at the EORTC-NCI-ASCO Annual Meeting on Molecular Markers in Cancer (November 2007, Brussels, Belgium) and as a podium presentation at the AACR Annual Meeting 2008 (April 2008, San Diego, California, USA).

**Statement of Translational Relevance**

The influence of APC I1307K mutations on CRC risk is the main controversial issue concerning the APC gene. Although there are previously published reports addressing this issue and the role of the APC I1307K variants, all studies singularly lack the power to estimate the correlation between genotype and phenotype in a meaningful way. By trying a new approach in this study, we have discovered an additional piece of the puzzle, necessary to provide a definitive answer. Accordingly, we have important results that demonstrate a clear and relevant correlation of the risk of CRC with APC I1307K and FOXO1A. Our findings could be helpful in identifying patients at an increased risk of developing CRC.

**Address for correspondence:**
Salvatore Pucciarelli
Clinica Chirurgica II, Dipartimento di Scienze Oncologiche e Chirurgiche
Università di Padova
Via Giustiniani 2
35128 Padova, Italy
e-mail: puc@unipd.it
REFERENCES


