DNA Sequence Profiles of the Colorectal Cancer Critical Gene Set KRAS-BRAF-PIK3CA-PTEN-TP53 Related to Age at Disease Onset

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Abstract

The incidence of colorectal cancer (CRC) increases with age and early onset indicates an increased likelihood for genetic predisposition for this disease. The somatic genetics of tumor development in relation to patient age remains mostly unknown. We have examined the mutation status of five known cancer critical genes in relation to age at diagnosis, and compared the genomic complexity of tumors from young patients without known CRC syndromes with those from elderly patients. Among 181 CRC patients, stratified by microsatellite instability status, DNA sequence changes were identified in KRAS (32%), BRAF (16%), PIK3CA (4%), PTEN (14%) and TP53 (51%). In patients younger than 50 years (n = 45), PIK3CA mutations were not observed and TP53 mutations were more frequent than in the older age groups. The total gene mutation index was lowest in tumors from the youngest patients. In contrast, the genome complexity, assessed as copy number aberrations, was highest in tumors from the youngest patients. A comparable number of tumors from young (<50 years) and old patients (≥70 years) was quadruple negative for the four predictive gene markers (KRAS-BRAF-PIK3CA-PTEN); however, 16% of young versus only 1% of the old patients had tumor mutations in PTEN/PIK3CA exclusively. This implies that mutation testing for prediction of EGFR treatment response may be restricted to KRAS and BRAF in elderly (≥70 years) patients. Distinct genetic differences found in tumors from young and elderly patients, whom are comparable for known clinical and pathological variables, indicate that young patients have a different genetic risk profile for CRC development than older patients.

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Introduction

The incidence of colorectal cancer (CRC, MIM#114500) has increased in the western world including Norway during the last 50 years [1]. CRC is typically found in elderly people with a median age at onset of 70 years, and only about five percent of all cases are diagnosed in patients younger than 50 years of age. Hereditary syndromes such as familial adenomatous polyposis (FAP, MIM#175100) and Lynch syndrome (HNPCC, MIM#120455) are found in less than five percent of all CRCs [2]. Although early onset of disease is generally accepted to be indicative of a potential genetic risk, most young at onset cases are regarded as sporadic as no known genetic predisposition is found [3]. Few studies have focused on the somatic tumor development in young patients with no known inherited syndromes [4–6].

Receptor tyrosine kinase (RTK) signaling is essential for maintaining the metabolism, proliferation, survival and motility of a cell [7]. Thus, errors in components regulated by receptor tyrosine kinases (RTKs) are commonly observed in human cancers [8,9]. The oncogenes KRAS (HGNC:6407), BRAF (HGNC:1097), and PTEN (HGNC:3895) and the tumor suppressor gene PIK3CA (HGNC:49588) are all affected in response to cytokines, growth factors and hormones signaling through RTK.

Both KRAS and BRAF have been shown to have activating mutations in ~70% of CRCs [10], leading to autonomous ERK-signaling [11,12]. Mutations in components of the PI3-kinase...
pathway have been reported as mutated in ~40% in CRC [13], and analyses of the genomic landscape of CRC tumors have shown the PI3K pathway to be affected in a statistically significantly manner [14]. In two of these components, PTEN and PIK3CA, mutations result in constitutive activation of the PI3K pathway by accumulation of phosphatidylinositol (3,4,5) triphosphate (PIP3), which then catalyzes the phosphorylation of AKT. The activated serine-threonine kinase AKT regulates a broad range of target proteins involved in a variety of downstream signaling pathways [15]. Among AKTs downstream targets is the tumor suppressor gene TP53 (HGNC:11998), a transcription factor that integrates information from many different types of cellular stress, and execute downstream responses appropriate for the given input [16]. TP53 is found mutated in about half of all colorectal carcinomas [17].

MSI status in the primary tumor has prognostic impact in CRC patients [18–20]. KRAS and TP53 status has been associated with clinical endpoints, the former only with weak association in larger series and the latter only if subgroups of mutations are considered [21–23]. Recently, one study has shown that PIK3CA mutations may carry prognostic information in tumor stages I–III [24]. This type of information has not been published for PTEN mutations.

The metastatic disease treatment targeting EGFR is found to be efficient only if KRAS and BRAF are not mutated [25]. However, even among the patients with wild type KRAS and BRAF not all respond to this therapy [26,26]. Lately, it has been proposed that this may be due to mutations in PIK3CA and PTEN, i.e. only quadruple mutation negative tumors will respond to treatment [26–29].

In the present study we have compared the DNA sequence mutation status of the genes KRAS, BRAF, PIK3CA, PTEN, and TP53 in tumor samples from patients younger than 50 years at diagnosis and without known hereditary syndromes, and stratified according to MSI status and elderly CRC patients.

### Results

Clinicopathological data for the whole series (n=181), divided into three age groups, are presented in Table 1.

#### Mutations in Colorectal Cancer

Clinicopathological data for the whole series (n=181), divided into three age groups, are presented in Table 1.

| Age at onset of disease | <50 years, n = 45 (%) | 51–70 years, n = 67 (%) | >70 years, n = 69 (%) |
|-------------------------|----------------------|------------------------|----------------------|
| **Min**                 | 27                   | 51                     | 71                   |
| **Max**                 | 50                   | 70                     | 93                   |
| **Mean**                | 41                   | 61                     | 80                   |
| **Sex**                 |                      |                        |                      |
| **Female**              | 23 (51)              | 31 (46)                | 36 (52)              |
| **Male**                | 22 (49)              | 36 (54)                | 33 (48)              |
| **Tumor location in the large bowel** |                  |                        |                      |
| **Right**               | 12 (27)              | 29 (43)                | 33 (48)              |
| **Left**                | 16 (36)              | 15 (22)                | 20 (29)              |
| **Rectum**              | 17 (38)              | 23 (34)                | 16 (23)              |
| **Stage**               |                      |                        |                      |
| **I**                   | 5 (11)               | 14 (21)                | 16 (23)              |
| **II**                  | 16 (36)              | 22 (33)                | 28 (41)              |
| **III**                 | 17 (38)              | 22 (33)                | 22 (32)              |
| **IV**                  | 7 (16)               | 9 (13)                 | 3 (4)                |
| **Mutations**           |                      |                        |                      |
| **KRAS**                | 13 (30)              | 20 (30)                | 24 (35)              |
| **BRAF**                | 3 (7)                | 12 (18)                | 13 (19)              |
| **PIK3CA**              | 0                    | 5 (8)                  | 3 (4)                |
| **PTEN**                | 8 (18)               | 7 (10)                 | 11 (16)              |
| **TP53**                | 29 (64)              | 26 (39)                | 38 (55)              |
| **MSI**                 | 6 (13)               | 13 (19)                | 14 (20)              |

| *Impaired PTEN: Combined results from sequencing and MLPA. |
| *Percentages are calculated based on the number of patients with successful analyses. |

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**Table 1.** Clinicopathological features and gene mutation status of colorectal carcinomas for each of the three age groups.
respectively ($P=0.04$). Furthermore, among the six MSI tumors in the <50 group only half of the samples carried mutations in the genes analyzed, whereas among MSI tumors in the other two age groups, all samples displayed one or several mutations.

**KRAS** mutations were found in 57 (32%) tumors in the total series. The mutation frequencies were slightly different between the age groups, Table 1. Most of the mutations were found in codons 12 (68%) and 13 (28%) independent of age group. The codon 61 mutations were only found in samples from the >70 age group.

**KRAS** and **BRAF** mutations were mutually exclusive in the total series. The frequency of tumor mutations in either **KRAS** or **BRAF** increased with patient age, both when comparing age groups and with age as a continuous variable.

Surprisingly, none of the tumors in the <50 age group had **PIK3CA** mutations, whereas five (7%) and three (4%) were found in the 51–70 group and >70 group, respectively.

Eighteen **PTEN** mutations were identified in seven of nine exons (no mutations were observed in exon 4 and 9). The mutations were observed in all age groups, as was the case for **PTEN** deletions. Deletions detected by MLPA were found in eight percent of the samples in total, and coexistence of deletion and alterations detected by sequencing were found in three tumors. Overall, 14% of the tumors had indication of impaired **PTEN** function. The frequency of **PTEN** aberrations showed no statistically significant difference between the age groups; 10%, 10% and 16% in <50 group, 51–70 group and >70 group, respectively.

The number of **TP53** mutated tumors differed among the three age groups ($P=0.02$). This difference was also significant when analyzing MSS tumors only, ($P=0.03$). Mutations were most prominent in the DNA binding domain, encoded by exons 5–8, and only about five percent of the mutations were observed in exon 4 and exon 11. Moreover, in the <50 age group, **TP53** mutations and **MSI** were mutually exclusive, and inversely correlated in the 51–70 and >70 groups, respectively.

**Genome complexity and sequence mutations**

For a subset of the samples (n=41) a high resolution dataset of copy number changes obtained by array comparative genome hybridization data (aCGH) was available (Roche NimbleGen, 385 000 oligo probe array) [30]. As previously published [30], we found the <50 group to have a significantly greater number of aberrations than the >70 group, even though the total portion of the genome with aberrations was similar in the two groups, Table 2. A difference in complexity was also found at the gene level, assessed as mutation index: in the older group each patient had 1.5 mutations on average, compared to 1.0 in the younger age group. The distribution of tumor stages was somewhat skewed between the two groups. However, neither the percentage of genomic aberrations, nor number of mutations differed among the tumor stages.

**Clinical associations**

No difference in survival was found comparing the age groups, even when adjusting for tumor stage at diagnosis. There was a trend that the proportion of rectal tumors decreased with increasing age ($P=0.08$), and the younger patients tended to have a more advanced tumor stage than elderly patients. The prevalence of stage III–IV tumors was 54%, 46%, and 36% in the <50, 51–70 and >70 groups, respectively.

In the total sample set, tumor stage was the most powerful prognostic variable with regard to three-year overall survival, ($P<0.001$). In univariate analysis, patients with **TP53** mutated tumors had poorer survival rates than patients with wild-type **TP53**, 938±31 days vs. 1016±23 days ($P=0.04$), respectively. However this difference was not significant when correcting for tumor stage. **TP53** mutations were of higher prognostic significance in right-sided tumors, 1051±26 days vs. 883±65 days for wild type and mutated **TP53**, respectively ($P=0.005$).

Among patients in the youngest age group, those with **KRAS** mutation had significantly shorter survival than patients with **KRAS** wild-type samples, 841±101 days vs. 1033±55 days ($P=0.04$), respectively. None of the other mutational parameters showed any statistically significant difference with regard to survival, neither within nor between age groups.

**Discussion**

We found that the mutation status of a known cancer gene set varies in CRC patients depending on age at onset of disease, and
provide further evidence of differences in the somatic development of tumors in young and elderly patients.

Microsatellite instability status

In all age groups, MSI was statistically significantly associated with right-sided tumors, as expected [18,19,31,32]. The frequency of MSI in the present series was somewhat higher than reported elsewhere [33,34], which is best explained by a random excess of right-sided tumors in the total series. However, for the group of patients younger than 50 years at diagnosis the frequency observed were slightly lower than expected [33], which partly could be explained by the exclusion of patients with known CRC syndromes in this study.

In patients younger than 50 years only 50% of the MSI samples were mutated in one or more of the genes analyzed, whereas in patients older than 50 all MSI samples displayed gene alterations. Additionally, BRAF mutations were not statistically significantly associated with MSI in the <50 years group, contrary to what was expected [35]. The mutation in BRAFV600E is observed to induce down-regulation of essential DNA repair genes [36], with potential to induce MSI in the cell, explaining this correlation. As BRAF mutations are not observed in tumors from patients with Lynch syndrome [37], we cannot exclude that MSI/BRAF wild-type samples in the present series are from clinically undetected Lynch syndrome patients.

Gene mutations

Mutations frequencies in KRAS, BRAF, PIK3CA, PTEN, and TP53 were found to be within previously reported range supporting the representativeness of the series [38].

The coexistence of BRAF and KRAS mutations is presumed to be incompatible with proliferation, hence a negative selection for concurrent existence of both mutations [12]. We did confirm an inverse correlation between mutations of these two genes [39]. In addition, an increasing number of tumors with either KRAS or BRAF mutation was observed with increasing age, in agreement with a recent report on young patients with colorectal cancer [6].

In the total series, tumors displaying BRAFV600E were typically TP53 wild type and PTENmutated (Table S1), which was also the case for tumors within the >70 age group. Thus, “BRAFV600E, TP53wt, PTENmut” may be regarded as a feature of genuine sporadic tumors.

In comparison to the reported 14% frequency of PIK3CA mutations in colorectal carcinomas in the COSMIC database [38], the current series shows only 4% mutations. This may partly be explained by the fact that the current series is enriched with samples from young patients, and their tumors did not have PIK3CA mutations. However, this latter observation is in contrast with two previous studies [6,40] that reports two/one patients younger than 50 years with PIK3CA mutations. The different methodologies used, sample type, formalin-fixed or fresh frozen tissue, and the size of the series may partly explain this difference in mutation frequency among the studies. Mutation in either the oncogene PIK3CA or the tumor suppressor gene PTEN may lead to an accumulation of PIP_3, and downstream AKT activation. However, since a sequence change in PTEN imply one remaining wild type allele, additional activating mutation in the same pathway may be beneficial for the tumor cells [41]. We found 32 tumors with alteration(s) in each of PIK3CA and/or PTEN, including seven tumors with several PTEN aberrations. The whole coding sequence of PTEN has been explored [42–48], but none of the studies have investigated tumors from young patients specifically. To our knowledge this is the first report combining PTEN whole coding sequence data with the gross deletion pattern in CRC from different age groups.

The TP53 mutation frequency was significantly higher in the <50 age group, than in the other age groups, which is best explained by the higher frequency of left sided and rectal cancers in the <50 age group. As expected, TP53 mutations were associated with MSS tumors, and the significant difference between the age groups was still valid when analyzing MSS tumors only.

A mutation index was calculated and tumors from elderly patients were found to have accumulated more gene sequence changes than the ones from young patients (1.5 vs. 1.0) which might indicate a time difference in tumor development.

Distinct genetic make-up of tumors from young and elderly patients

From aCGH data on a subset of 41 samples, we observed that patients younger than 50 years had significantly more aberrations in their tumors than patients >70 years old, i.e. a higher degree of genomic complexity. Contrary, as mentioned above, the tumors from elderly patients had the highest gene mutation index, even though this group included fewer advanced stages. Taken together, one may speculate that among the young patients there are carriers with genetic predisposition, affecting genes encoding proteins that ensure correct chromosome segregation during mitosis, rather than specific pathways.

Bardelli et al. [29] reported that patients with metastatic disease that are “quadruple negative” in the KRAS-BRAF-PIK3CA-PTEN gene set have the highest probability of response to anti-EGFR therapies. However, currently only KRAS testing is recommended prior to decision with regard to such treatment, and BRAF testing is optional [49].

In the present series of primary cancers, mutation frequencies in these four genes were equally distributed between all tumor stages. Therefore, we have in the following hypothesized the number of potential responders based on the whole dataset (Figure 2). When considering tumors with impaired PTEN and/or mutated PIK3CA exclusively, the youngest age group displayed 16% (n = 7) mutations, compared to 1.4% (n = 1) in the oldest age group. This implies that for older patients, KRAS and BRAF mutation status alone will reveal 98% of the patients unsuitable for anti-EGFR therapy, as compared to 71% in patients <50 years. The effect of mutations in PIK3CA and PTEN are debated with regard to anti-EGFR therapy [26,28,29]. Notwithstanding, if their mutational status does not influence on the effect of anti-EGFR treatment, the younger patients will be expected to have an even larger proportion of potential responders to this type of treatment than the older patients (Figure 2).

Association to clinical data

The consecutive sample series of colorectal carcinomas from a single hospital showed overall distribution of clinicopathological data as expected for a Norwegian cohort [1]. The proportion of rectal cancers decreased with increasing age at onset, whereas the opposite trend was seen for right-sided tumors, as reported by others [50,51]. Furthermore, a larger part of the patients diagnosed before age 50 years had more advanced disease stage compared to the older age groups. This may reflect that CRC is unexpected in young adults and symptoms are neglected both by the patient and the physician [52]. Reports of young colorectal cancer patients differs with regards to aggressiveness of the disease, and hence outcome [3,32,53]. However, in the present study, the three-year overall survival was equal in the three age groups, even when adjusting for tumor stage. This might indicate a more aggressive disease in young patients, as older people are expected to have a higher mortality rate. Furthermore, the observed higher degree of genomic
complexity in tumors from young versus elderly patients also may be indicative of a difference in aggressiveness. It should be noted, that the young patients included are not carriers of known colorectal cancer syndromes according to clinical criteria only.

The prognostic and predictive value of TP53 is still controversial [54]. In our dataset patients with TP53 mutations had significantly shorter survival than wild type patients. Furthermore, for patients with right-sided or MSI tumors TP53 mutations showed even greater prognostic value, in line with results reported by the TP53-CRC collaborative study group [55].

In a recent study, PIK3CA mutations were found to serve as surrogate markers for poor survival in colorectal cancer patients [24]. As few PIK3CA mutations were observed in our sample set, we could not confirm these results. However, mutations were found only in samples from older patients, and were not present in patients with distant metastases, and only few in stage III patients. Thus, our data suggest that PIK3CA mutations are important for tumorigenesis among elderly patients, but do not serve as a marker for the ability to metastasize.

Conclusion
We have found distinct differences in the genetic make-up of carcinomas from young and elderly patients. The tumors from young patients whom are not carriers of known hereditary CRC syndromes have less gene mutations, but more copy number aberrations across the genome. These data suggest that some young patients may have a potential increased risk for cancer caused by alterations in gene(s) involved in maintaining correct chromosome segregation.

Materials and Methods

Ethics statement
Written informed consent was obtained from all subjects included. The research biobanks are registered according to national legislation and the research studies are approved by the Regional Committee for Medical Research Ethics (REK South-East: 1.2005.1629; REK South, 2003: S-02126).

Patients and tumor samples
A total of 181 patients were included in the study, i.e. a consecutive series of 132 patients enriched with a series of 49 patients with disease at young age (<50 years). Each series are described in the following paragraphs. Clinical data were collected prospectively. Death data were retrieved from either the hospital records or from the Norwegian Population Registry. The clinical and pathological data of these two series are shown in Table S3.

Figure 2. Graphic representation of patients with hypothesized response to EGFR-targeted therapy. Percent-wise distribution of aberrations for KRAS, BRAF, PIK3CA and PTEN and their combinations, in the <50 age group and >70 age group. Patients negative for all mutations (quadruple negative) are potential responders to anti-EGFR-therapy. (Modified from Bardelli et al.[29])

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Consecutive series (n = 132). Patients undergoing elective resection of colorectal adenocarcinoma in 2005–2007 at Oslo University Hospital, Aker, Oslo, were included. Patients with FAP or Lynch syndrome were excluded based on clinical criteria. Tumor tissue was sampled immediately after resection of the specimen and instantly frozen in liquid nitrogen. All tissue specimens were evaluated by a pathologist to establish differentiation grade of the carcinoma and tumor cell content.

Early onset series (n = 49). Samples from a biobank from a multi-hospital study (INFAC-study - individuals with familial risk for cancer), including Oslo University Hospital, Aker, Oslo and five other hospitals in the same geographical region of Norway enrolled from 2003–2008, were included in the present study. The inclusion criteria in the INFAC-study were age <55 years, excluding those who fulfilled criteria of all known CRC syndromes. Tumor tissue and corresponding normal mucosa samples were taken from the resected specimen in the operation theatre and promptly incubated in RNAlater. The tissue was transferred to new tubes after 36–72 hours, and stored at minus 80°C until use. DNA from normal and cancerous tissues was extracted, and used for analysis.

Samples included in CGH analysis. For a subset of the patients (n = 41) data from aCGH analyses were available. This subset included 24 patients younger than 50 years, and 17 older than 70 years at primary diagnosis. Clinical and mutational data for these patients are presented in Table S2.

Grouping of patients according to age. Patients from the early onset series were included to enrich the number of early onset patients in the total sample set. Patients from the early onset series were selected to match patients younger than 55 years in the consecutive series with regard to gender, median age, tumor stage and localization, Table S3. Thereafter, samples from the two series were combined and divided into three age groups; under age 50 years, 51 through 70 years and over 70 years, respectively. The mean age at primary diagnosis was 70 years in the consecutive series, and that was chosen as cutoff for the oldest age group. The cutoff for the youngest age group was set at 50 years, since early onset may be due to inherited genetic factors, and only ~5% of all CRC are diagnosed before 50 years of age. Tumors in the colon proximal to the splenic flexure were defined as right-sided: those in the remaining colon were defined as left-sided whereas rectum was defined as 15 cm from the anal verge when measured with a rigid proctoscope. Localized disease was defined for UICC/AJCC stages I and II and regional disease for stages III and IV.

Microsatellite instability analysis
For all patients, tumor and corresponding normal tissue or blood was analyzed using the Bethesda markers [36]. The analysis was performed as described by Wu et al.[37]. High degree of microsatellite instability (MSI-H) in tumor DNA, as compared to corresponding normal DNA, was defined if two or more markers showed aberrant peak profile after fragment analysis. If both the normal sample and the tumor sample from a patient showed the same pattern for all markers, the tumor was regarded microsatellite stable (MSS).

Mutation analysis for KRAS, BRAF, PIK3CA, PTEN and TP53
For TP53 and PTEN, the total coding sequence was amplified in multiplex PCR reactions, using multiplex PCR kit as recommended by the vendor (QIAGEN, GmbH, Hilden, Germany) [30,60]. Singleplex PCR reactions using HotStar Taq (QIAGEN) were used to amplify PIK3CA, KRAS and BRAF amplicons [10,59,61]. In PIK3CA exons 9 and 20 were amplified, in KRAS, exons 2 and 3, including the frequently mutated codons 12, 13 and 61, and in BRAF exon 15, including the mutation in codon 600, were amplified. Primers, running conditions and fragment details are described in Table S4.

PCR products were purified using Sephadex columns (Millipore, Billerica, MA, US and GE Healthcare, Chalfont St.Giles, UK) prior to incorporation of dye labeled ddNTPs and sequencing on the 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, US) [30]. In cases where a mutation was detected, a new independent PCR product was subjected to sequencing to confirm the result.

Multiplex ligation-dependent probe amplification (MLPA) of PTEN
Salsa MLPA kit P225-B2 PTEN (MRC Holland, Amsterdam, Netherlands) were used according to instructions from distributor. In total, 25 probes cover the PTEN gene, a minimum of two probes for each of the nine exons. Also, 10 probes located elsewhere on chromosome 10, and additional 12 reference probes located on other chromosomes serves as controls. Amplified fragments were analyzed on a 3730 DNA Analyzer (Applied Biosystems). Raw data were analyzed with Coffalyzer® (MRC Holland) with default settings. Cutoffs of 1.2 and 0.8 were used for scoring of gains and losses, respectively, in addition to individual evaluation of the plot from each sample. This scoring is determined according to the following calculation: one copy gain or loss in 60% of the cells in a triploid tumor equals 1.2 and 0.8, respectively. At least two neighboring probes, located in the same exon, with concomitant gain or loss, were confined for assigning aberrant copy number in a region.

Statistical analyses
Clinical variables were tested for associations with mutational results for all genes analyzed, and all genes were tested for inter-relational associations. All statistical analyses were performed using the software package PASW 17.0 (SPSS Inc., Chicago, IL, US). Fisher's exact test was used for cross-tabulated variables. Student T-test was performed when calculating the relationship between a continuous variable and a categorical variable. Survival analyses were performed using the Kaplan-Meier life table analyses with three years overall survival, and log rank test used to compare the survival curves. For all statistical tests, a two-tailed P<0.05 was considered significant.

Supporting Information
Table S1 Relation of mutational and clinical data on tumors from included patients. NS = not significant, NA = not applicable. All P-values <0.20 are shown in the table, and P<0.05 is highlighted in bold.
Found at: doi:10.1371/journal.pone.0013978.s001 (0.03 MB XLS)
Table S2 Details of clinical and mutational data from KRAS, BRAF, PIK3CA, PTEN and TP53. * F = female, M = male. # patients included in Table 2. $ results from MLPA.
Found at: doi:10.1371/journal.pone.0013978.s002 (0.05 MB XLS)
Table S3 Clinical characteristics in different age groups. Distribution of clinical data in the total consecutive series, young in consecutive series (patients under age 55 years), early onset series (<55 years), and in the total series of patients. Found at: doi:10.1371/journal.pone.0013978.s003 (0.03 MB XLS)

Table S4 PCR and primer details for genes analyzed. #M13 forward primer (TGTTAAACGAGGCAGCT). #M13 reverse primer (CAGGAAGACGCTATGACC). Found at: doi:10.1371/journal.pone.0013978.s004 (0.03 MB XLS)

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Conceived and designed the experiments: MB SAD RAL. Performed the experiments: MB SAD TA THA˚. Analyzed the data: MB SAD TA THA˚. Contributed reagents/materials/analysis tools: MAM MV TM OHS AB IM TF OM AH OS AN ETE RA˚. Wrote the paper: MB SAD.
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