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A Single Nucleotide Polymorphism in the MGMT Gene is a Novel Prognostic Biomarker in Patients with Metastatic Colorectal Cancer

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RM, JBN and AS did the sample preparation and sequencing of the patient DNA samples by different technologies namely direct sequencing, pyrosequencing and sequenom. Author DMR supervised the overall sequencing technology and primer design for sequenom. Authors AS, AM and MB compiled the patient data. Authors JBN and SG did the sequenom data analysis. Authors SA and SG performed the multivariate analysis. Authors RM and SG prepared the manuscript. Authors TAA and JMM provided critical scientific inputs and experiment planning. All authors read and approved the final manuscript.

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ABSTRACT

Background: Discovery of novel biomarkers of prognosis and drug response remains an elusive, yet critical goal. Thus, accurate and rapid screening of an array of pertinent mutations/SNPs is an essential step in cancer management.
Methods: Using a high-throughput multiplex PCR microarray technique, we simultaneously screened the mutational status/SNP of 32 hotspots in multiple genes for metastatic colorectal cancer (mCRC) from 126 formalin fixed paraffin embedded samples from 78 patients. The efficacy of the technology was validated by cross-comparison with conventional Sanger sequencing and

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pyrosequencing. The clinical outcome was corroborated to the mutational status to determine prognostic and predictive significance of the 32 loci.
Results: In a statistically robust multivariate model, patients with the TT genotype of the MGMT gene (rs1625649) enjoyed a significantly longer survival (61.8 months) when compared to those with GG or heterozygous GT genotype (29.3 months) [HR 0.30; 95% CI: 0.10-0.89, P= 0.03], with a 70% reduced risk of death from mCRC.
Conclusion: The rs1625649 SNP within MGMT is a novel and potentially valuable prognostic biomarker for mCRC patients.

Keywords: Metastatic colorectal cancer; MGMT; sequenom; predictive; prognostic.

1. INTRODUCTION

Identification of novel prognostic and predictive biomarkers is purported to improve the classification and management of metastatic colorectal cancer (mCRC), a disease with a median survival of 24-30 months [1]. Several studies have investigated molecular markers predictive of response to chemotherapeutic agents enabling individualized therapy. However, with the exception of *KRAS* mutation status being predictive for non-response to anti-EGFR antibody treatment [2,3] none of these markers has been introduced into routine clinical practice. In terms of prognostic markers, the loss of the mismatch repair (MMR) genes such as MLH1 has been suggested to be a marker of better prognosis [4] especially in stages II and III, and possibly predictive for worse outcome when patients with stage II are treated with 5-FU adjuvant therapy [5]. Although the etiology of different cancers vary significantly, cancer is a disease of altered DNA structure and function [6], and in general, cancer is not a consequence of single gene alteration but rather a combined effect of several genetic aberrations [7]. The knowledge of the mutational/SNP status of a wide spectrum of prognostic and predictive markers simultaneously can better guide the clinicians in making personalized management decisions.

The feasibility of rapid identification of the mutational/SNP status of multiple hotspots in a time efficient manner from a sparsely available clinical sample is difficult to achieve by traditional sequencing technologies. The successful development of high throughput mass spectrometry-based genotyping technology generated by the Sequenom platform provides a cost-effective methodology that can simultaneously detect frequent and infrequent mutations in a large number of samples utilizing small quantity of DNA (5-10 ng). Furthermore, it has been reported that the mass spectrometric method is more sensitive than traditional Sanger sequencing, and is highly concordant with commonly utilized sequencing techniques [8,9].

We aimed to utilize the rapid diagnostic sequenom screen for a broad spectrum of mutations and single nucleotide polymorphisms (SNPs) in mCRC patients in an effort to identify novel biomarkers. To do so, we designed a study where in DNA was isolated from formalin fixed paraffin embedded (FFPE) samples, screened for 32 hotspots that has been previously reported to have some correlation to mCRC (Supplementary Table 1) in a time efficient manner, and analyzed in relation to clinical outcomes, with a focus on overall survival (OS). The data was then analyzed in a robust statistical multivariate platform to ensure confidence of the findings.

2. MATERIALS AND METHODS

2.1 Patient Selection

Patients were those with mCRC treated at Montefiore Medical Center (MMC) 2004 and 2009 (Table 1), as published previously [10]. DNA was isolated from FFPE tissue from 126 specimens from 78 patients and was subject to sequencing by multiplex PCR technique. The protocol was duly approved by the institution's IRB committee.

The experimental method was carried out in accordance with the approved guidelines. Patient data was obtained from the MMC tumor registry database including demographic and survival information such as date of birth, diagnosis, and last contact (defined as either date of death or date of last follow-up), and ethnicity (categorized as Hispanics, non-hispanics (NH)-Whites and NH-Blacks). Additional clinical characteristics were extracted through retrospective chart review using Clinical Information System (Centricity® Enterprise, version 6.6.3) and Electronic Patient Folder (McKesson Information

Solutions LLC). Characteristics collected at baseline were: gender, site of primary tumor (colon vs. rectum), number of metastatic sites at diagnosis, diagnosis as stage IV disease vs. recurrence, tumor differentiation, number and type of chemotherapy received, and CEA at diagnosis. All patient information was de-identified and stored as a Microsoft 2007 Excel file that was encrypted and password protected. Overall survival (OS) was defined as time from diagnosis of metastatic disease to date of death or last follow up.

2.2 SNP Selection

The 32 loci selected for investigation were a combination of somatic and germ line SNP/mutations. Of these, 28 hotspots that were chosen have a plausible contribution in the metabolism of the standard chemotherapeutic drugs, and 5 hotspots for possible prognostic implication, with ERCC1 SNP common in both categories. The selected hotspots had an available rs numbers (SNP records/reference SNP ID or "rs#"; "refSNP cluster") making PCR based mass spectrometric analysis feasible. The 28 hotspots were grouped into 4 categories, namely: (a) resistance to anti-EGFR antibody therapy (14 hotspots, 13 somatic and 1 germline, in 4 genes) (b) oxaliplatin metabolism/resistance (5 SNPs in 5 genes) (c) 5-FU metabolism (7 SNPs in 5 genes) (d) bevacizumab metabolism (2 SNPs in 1 gene). Five genes namely MLH1, MSH2, p53, ERCC1 (common for oxaliplatin resistance and overall prognosis) and O-6-methylguanine-DNA methyltransferase (MGMT) were designated for a prognostic evaluation (supplementary Table 1).

2.3 High Throughput Sequencing Using the Sequenom Platform

SNP sequences or "rs number" were provided to Sequenom (www.sequenom.com) for assay design. The multiplexing was designed by Sequenom as were the primer sequences for the PCR reaction. The Un Extended Primers (UEP) specific for each rs number was ordered from Fisher Scientific (supplementary Table 2). Sequenom multiplexing was thus performed on 32 hotspots in 19 genes plexed together in 4 reactions containing 8 SNPs each. The PCR reaction was carried out using a touchdown protocol. PCR amplified samples were purified using shrimp alkaline phosphatase followed by single nucleotide extension using specific extension primers, and the products were analyzed by mass spectrometry (MALDI-TOF).

Ten hotspots relevant to anti-EGFR resistance were selected and sequenced by direct and pyrosequencing methodologies to validate the concordance.

Table 1. Patient characteristics

Clinical Characteristics	N=78
Age (median/range)	**59 (34-93)**
Gender % (no)	
Male	29.5 (23)
Female	70.5 (55)
Race % (no)	
White	21.8 (17)
Hispanic	32.1 (25)
Black	42.3 (33)
Other	3.8 (3)
Diagnosis % (no)	
Stage IV	47.4 (37)
Recurrence	52.6 (41)
Site of primary % (no)	
Colon	73.1 (57)
Rectum	26.9 (21)
Sites of disease % (no)	
Liver only	24.3 (19)
Other sites	75.7 (59)
Number of metastatic sites (median/range)	3 (1-6)
Differentiation % (no)	
Well	15.7 (11)
Moderately	54.3 (38)
Poorly	21.4 (15)
Unk	8.6 (6)
CEA at diagnosis of stage IV (median/range)	24.5 (0.5 – 5500)
Number of chemo lines % (no)	
1	20.5 (16)
2	39.7 (31)
3	33.3 (26)
4	4.5 (4)
No chemo	1.3 (1)
Received Biologics % (no)	
Yes	92.3 (72)
No	7.7 (6)
Received EGFR inhibitor % (no)	
Yes	91.1 (71)
No	8.9 (7)

Patient Characteristics (n=78). Patients were those with mCRC treated at Montefiore Medical Center (MMC) with anti EGFR therapy between 2004 and 2009, as published previously. DNA was isolated from FFPE tissue from 126 specimens from 78 patients and was subject to sequencing by multiplex PCR technique. Patient data was obtained from the MMCtumor registry database including demographic and survival information such as date of birth, diagnosis, and last contact (defined as either date of death or date of last follow-up), and ethnicity (categorized as Hispanics, non-hispanics NH-Whites and NH-Blacks). Additional clinical characteristics were extracted through retrospective chart review using Clinical Information System (Centricity® Enterprise, version 6.6.3) and Electronic Patient Folder (McKesson Information Solutions LLC). Characteristics collected at baseline were: gender, site of primary tumor (colon vs. rectum), number of metastatic sites at diagnosis, diagnosis as stage IV disease vs. recurrence, tumor differentiation, number and type of chemotherapy received, and CEA at diagnosis.

2.4 Data Analysis of PCR Multiplexing at 32 Hotspots

Results were provided in both genotype and allelotype format. All 4032 reactions were analyzed individually using the Typer 4 software from Sequenom by 2 authors (JN and SG). A "call" was made for each reaction defining the genotype by comparing the peak of each allele relative to the un-extended extension primer (UEP) peak. Decisions were aided by the position of each reaction on the yield v/s skew graph and the probability of each allele provided by the mass spectrometry graph.

2.5 Statistical Analysis

Descriptive statistics was used for patient characteristics. The Wilcoxon test was used to determine the P value. Kaplan-Meier method was used to report survival curves, and Wilcoxon test was used to compare them. A Cox proportional-hazard model was used to assess survival differences between patients with different SNP status in the MGMT gene. Survival modeling included the examination of univariate associations between survival and variables that were considered clinically relevant. Variables with a hazard ratio that had a p value of <0.2 in the univariate analysis were included in multivariate modeling. A priori decision was made to force the variables gender, age at diagnosis, and race into the model. Continuous variables were checked for linearity assumption, and when violated were categorized using clinical meaningful cut-points. One final model was selected which included the variable of interest (MGMT), gender, median age at diagnosis and other variables in which P values were <0.05 in the multivariate analysis. The final model was checked for interactions and confounding. Diagnostics of the final model was assessed by using graphical methods (log-log plots and observed vs. expected plots) and the goodness of fit test. All analyses were done using STATA 11.2 software (STATACorp LP).

3. RESULTS

3.1 Patient Characteristics

The clinical characteristics of the 78 patients are shown (Table 1). All patients had metastatic disease. The median age was 59, 47% were diagnosed with stage 4 cancer, 78% had received two or more chemotherapy regimens, and more than 90% had received biologic agents.

3.2 A SNP (rs1625649) in the MGMT Gene is a Novel Prognostic Marker

The rs1625649 SNP in the DNA repair gene, MGMT is a valuable prognostic marker in mCRC patients (Fig. 1). In a univariate analysis, patients with the TT genotype (12% of patients) had a median OS of 61.8 months, while those with homozygous GG or heterozygous GT had a median OS of 29.3 months (P=0.06). Further, in multivariate modeling, patients with the TT genotype had longer survival when compared to those with homozygous GG or heterozygous GT (HR 0.30; 95% CI:0.10-0.89, P= 0.03), after adjusting for known clinical prognostic parameters such as gender, race, age at diagnosis, number of metastatic sites, number of chemotherapy lines received and CEA at diagnosis (Table 2). Therefore, the patients with the TT genotype had a 70 % reduced risk of death.

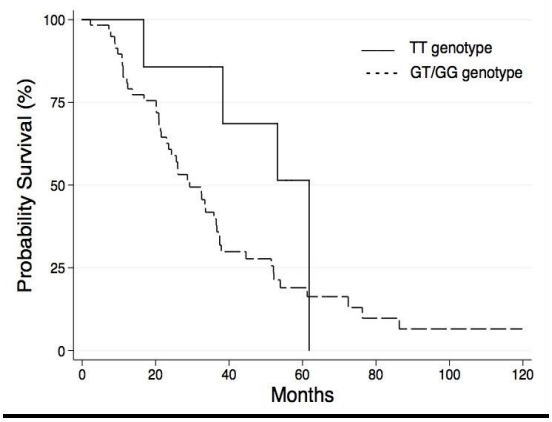

Fig 1. Overall survival based on MGMT SNP

A graphical representation of MGMT SNP status as a novel prognostic marker in CRC. MGMT SNP has significant contribution to the overall survival of CRC patients. In the multivariate model, the analysis was significant with a P value of 0.03(multivariate analysis). The patients who carry the TT genotype have a superior OS (61.8 months) in comparison to those with a GG or GT genotype (29.3 months), with a hazard ratio of 0.30, signifying a 70% lower mortality rate

3.3 Concordance of the Sequenom Platform with Sanger Dideoxy Sequencing and Pyrosequencing

The accuracy of SNP detection by multiplex PCR was determined by comparison to results obtained from the gold standard Sanger dideoxy

sequencing as well as pyrosequencing for 10 randomly selected hotspots. The mutation/SNP detected by pyrosequencing was considered standard and validated with direct and multiplexed techniques (supplementary Table 3). Of the randomly selected 10 hotspots the prevalence of SNP for KRAS codon 12 (C12_1W2_G12S/V/D/A/C); ACC (A,T,G) was the highest (22%) as determined by pyrosequencing and when compared to direct and multiplex sequencing a 93% and 92% concordance was documented respectively. Similarly, a 98% and 97.4% concordance rate was obtained between Sanger sequencing and PCR multiplexing for the PIK3CA and BRAF genes respectively. These results validate the specificity and sensitivity of the sequenom PCR multiplexing methodology.

Table 2. Multivariate modeling for OS

Variable	HR (95% CI)	P value
Gender	0.80 (0.41-1.53)	0.50
Age (dichotomized at median)	1.08 (0.60-1.93)	0.79
Race (White vs. Others)	4.72 (1.74-12.79)	0.002
Metastases (1 vs ≥2)	1.95 (1.04-3.66)	0.04
Lines of chemotherapy (1 vs ≥2)	0.39 (0.18-0.88)	0.02
CEA (dichotomized at median)	2.29 (1.21-4.32)	0.01
MGMT (TT vs GT/GG)	0.30 (0.10-0.88)	0.03

A Cox proportional-hazard model utilized to assess survival differences between patients with different SNP status in the MGMT gene. Survival modeling included the examination of univariate associations between survival and variables that were considered clinically relevant. Variables with a hazard ratio that had a p value of <0.2 in the univariate analysis were included in multivariate modeling. The final model was checked for interactions and confounding. Diagnostics of the final model was assessed by using graphical methods (log-log plots and observed vs. expected plots) and the goodness of fit test. All analyses were done using STATA 11.2 software (STATA Corp LP)

3.4 Prevalence of Mutations/SNP in the Studied Patient Population

The frequency of mutations and or SNPs as analyzed by PCR multiplexing in our patient cohort were compiled and compared with other reported similar studies (Supplementary Table 4). The prevalence of mutations/SNPs in KRAS, PIK3CA, GSTP1, MHL1, ERCC1, ERCC2/XPD, XRCC1 and ABCB1 in our patient cohort was similar to previous findings (http://www.mycancergenome.org/content/disease/colorectal-cancer). In contrast, the mutation rate of BRAF, PTEN and AKT in our patient population was lower than previously reported. Validation of the SNP frequency in CRC of MSH2, DPD, MTHFR, TS, MGMT, ABCG2, VEGF1154, VEGF 2578, CCND1 and FCGR3A were not possible due to non-availability of any comparable studies investigating these genes as documented in reliable cancer related databases namely COSMIC (catalogue of somatic mutation in cancer) website: http://www.sanger.ac.uk/genetics/CGP/cosmic/, http://www.mycancergenome.org/content/disease/colorectal-cancer, http://snpedia.com/index.php/SNPedia, and http://www.ncbi.nlm.nih.gov/snp.

3.5 Allelic Frequency in the Patient Cohort

The comprehensive multiplex PCR sequencing for all the 32 loci yielded 4032 possible calls. Of these, 3711 of the calls were successfully analyzed, with the overall call success rate of 92%. The frequency of heterozygous and homozygous alleles for the 32 hotspots is herein reported (supplementary Fig. 1). The top 4 heterozygous allele in order of highest to lowest in frequency are: (A) the low affinity immunoglobulin gamma Fc region receptor III-A encoded by the FCGR3A gene (CD16a cell differentiation factor) was heterozygous for 64 out of 78 patient samples, being the most abundantly heterozygous allele of all hotspots; (B) the MGMT gene encoding DNA lesion repair protein, O (6)-methyl guanine-DNA methyl transferase (42 of 78); (C) the glutathione S-transferase polymorphic gene (GSTP1) that functions in xenobiotic metabolism and plays a role in susceptibility to cancer (40 of 78); and (D) the tumor suppressor gene Tp53 with (36 of 78). The frequency of SNP in both the alleles (homozygous) was highest for ERCC-2/XPD genes being 47 out of 78 followed by 46 out of 78 for XRCC1 gene.

3.6 Comparison of SNP's in Primary versus Metastatic Tissue

There were 31 patients with primary and metastatic samples. Analysis of mismatch between primary and metastatic sites was determined both for germ line and somatic SNPs. A total of 13 somatic polymorphism/mutations were analyzed in these matched samples with 403 possible calls. Overall, 348 calls were successfully determined of which only 6

mismatches were observed, accounting for a 1.72% mismatch rate. The analysis covered 7 mutations in KRAS exon 2 (codons 12 and 13) and 2 in KRAS exon 3 (codon 61), PIK3CA (E542K and H1047R) and Braf (V600E) mutations. We found 4 mismatches in KRAS (1 sample with 3 metastatic lesions: 2 concordant with primary, and 1 discordant), 1 in PIK3CA H1047R, and 1 in Braf. We also analyzed the concordance rate of 18 SNPs in 30 matched samples (1 pair not analyzed - bad sample). Of the 540 calls analyzed, the mismatch frequency was 2.47%. Overall therefore, the genotype of metastatic lesions closely matched the genotype of the primary tumor for these loci.

4. DISCUSSION

Despite the unique features that distinguish each malignancy, the underlying processes of carcinogenesis involves DNA perturbation, and as a result, DNA repair mechanisms assume central importance in the defense against carcinogenesis. This is demonstrated by the fact that global defects in the DNA repair apparatus can cause inordinately high risks of malignancies in different tissues [6]. Identifying and incorporating mutations and/or SNPs with robust prognostic and predictive value in care of cancer patients is a necessary step towards personalized clinical care. The high throughput multiplex PCR based extension on microarray offers a robust platform capable of detecting multiple SNPs simultaneously in a rapid and cost effective manner in contrast to the cost ineffective, time consuming laborious techniques of direct and pyrosequencing.

Our aim was to utilize the robust technology of multiplex PCR sequencing to simultaneously screen multiple pertinent mutations/SNP in a rapid manner that could be applied towards patient management soon after the patient presents with the disease. In the current study we also validated the concordance and efficacy of the sequenom technique to the well-established dideoxy and pyrophosphate based sequencing methods. The concordance rate was determined to be greater than 92% compared to Sanger direct sequencing and pyrosequencing for 10 hotspots (supplementary Table 2) giving us the confidence in the future success of the comprehensive genetic diagnostics of the disease.

We report for the first time, that the DNA repair gene, MGMT, has a prognostic role in mCRC. In our population, the patients who carry the TT genotype have an superior OS (61.8 months) in comparison to those with a GG or GT genotype (29.3 months), with a HR of 0.30, signifying a 70% lower mortality rate. Interestingly, while a search for prognostic markers for mCRC has been ongoing for decades, none has been established with certainty, including loss of the MMR proteins, which has been elusive as far as metastatic disease is concerned [11,12]. In fact, two excellent reviews, spanning a decade apart agonize over the lack of a single well established prognostic biomarker for mCRC [13,14]. Further, the loss of MMR (or MSI positive status) and LOH 18q were considered as category IIB as a prognostic marker, indicating that these have shown promise in multiple studies but lack sufficient data for inclusion in routine clinical practice [14]. Our current report therefore clearly presents a novel finding of a potential biomarker that ought to be carefully investigated, and in fact may turn out being the first validated prognostic biomarker ever for mCRC.

While this is the first report of a SNP in the MGMT gene as a potential prognostic biomarker in mCRC, there are sufficient data that methylation in the MGMT promoter is a positive predictive biomarker in patients with glioblastoma, when treated with temozolamide, an alkylating agent [15]. Furthermore, a recent population based study revealed that a SNP in MGMT, rs2296675 was significantly associated with overall cancer risk [per minor allele odds ratio (OR) 1.30 (95% confidence interval 1.19–1.43; $p=4.1 \times 10\text{-}8$) [6]. Although the reported SNP was different from the one in our current study, the fact that similar observation was made for an alternate SNP of the same gene validates our findings with confidence. One research paper that has studied MGMT SNP has identified an association between one particular SNP, rs12917 and degree of promoter methylation and MGMT expression mCRC [16]. Since MGMT's role is to fix DNA damage, one may speculate that low expression may lead to accumulation of DNA damage leading to development of cancer and early mortality. One potential future direction of our current finding is to study MGMT promoter methylation and gene expression in our patient population.

The availability of tissue to test for KRAS mutations is many a time limited to a primary or a metastatic lesion, and is critical especially when anti-EGFR therapy is being considered. Our finding on the high degree of concordance

between the primary and metastatic lesions provides comfort to the practicing clinician, and further demonstrates that either tissue is sufficient and accurate to make therapeutic decisions. Presence of a KRAS mutation in either the primary or metastatic lesion may be considered to withhold anti EGFR therapy in these patients. While the number of patients initially may appear to be low, the fact that the identified SNP (rs1625649) in the MGMT gene was significant in a robust multi variate modeling system gives us the confidence that it will serve as a valuable prognostic marker in mCRC. It is relevant to the context that one of the earliest papers that identified KRAS mutation as a potential marker of resistance to anti-EGFR therapy was a study with only 30 patients [14,17]. Since then larger datasets have indeed been reported. Similarly, we are confident that our data can be used to incite large scale analysis of the SNP status in groups that have ready access to patient's DNA and clinical history and outcome, such as the US cooperative groups.

We concede that this study has certain limitations. Most importantly, we recognize that the low number of patients is one potential limitation of our study. We have nevertheless discovered a potential prognostic marker using a rigorous statistical multivariate analysis. In spite of this limited sample size, using a multivariate model, the prognostic significance of this particular SNP remains statistically significant. Another limitation is that we have assessed SNP/mutations using the sequenom platform, and have assessed 32 hot spots in 20 genes. With improvement in technology, one can perform complete genome sequencing or next generation sequencing and potentially detect further prognostic and predictive markers. That is perhaps the next phase of a project such as ours.

Our findings should prompt large scale analysis by the cooperative groups that have access to large number of patient samples along with the clinical data, to further validate our findings. A major strength of the study is that being a single center study, all data has been verified and genomic analysis conducted at our large well experienced genomic facility. Importantly, in our multivariate model, we have incorporated the entire list of category-I prognostic biomarkers as suggested by the excellent review [13]. Such analysis utilizing multiplex sequencing technology will likely be much less time consuming and can reveal massive amounts of information about the disease and better identification of prognostic and predictive biomarkers.

5. CONCLUSION

The rs1625649 SNP within the MGMT gene is a novel and potentially valuable prognostic biomarker for patients with metastatic colorectal cancer. These novel findings should be validated and replicated in studies with larger datasets of patients and available tissue.

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ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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