Colorectal carcinomas from Middle East. *Molecular and tissue microarray analysis of genomic instability pathways*

To the Editor

The interpretations by Bavi et al of familial cases of colon cancer in Saudi Arabia (not in the Middle East) warrant further discussion. First, we congratulate the authors for this comprehensive and complex study, and agree with them that "the findings in this paper will pave the way for future studies". However, we have a number of significant concerns. The familial conditions associated with microsatellite instability and fulfilling the revised Bethesda criteria are autosomal dominant, and should be unrelated to consanguinity.[2,3] There are other types of familial colon cancer that may be associated with a high degree of consanguinity, such as multiple colorectal adenomatous polyps (MAP)[4] associated with biallelic inactivation of the MYH gene, and "familial colorectal cancer type X".[5] These 2 conditions may have family history consistent with hereditary non-polyposis colorectal cancer (HNPCC), however, they do not have evidence of mismatch repair (MMR) gene mutations and microsatellite instability. Other rare, high penetrance recessive alleles might well exist, and could be detectable by high-density single nucleotide polymorphism (SNP) haplotyping. Probably the explanation for the higher frequency of these cases in the Saudi population (providing it is real, although we did not see a statistical proof) would be a "founder" effect. The authors mentioned this possibility, but they ignored it in the abstract and in most of the discussion. Events that increase the likelihood of a founder mutation, like the rapid population growth of Saudi Arabia, should be considered. When mutations in the *MLH1, MSH2, MSH6, PMS2* genes cause Lynch syndrome (previously called HNPCC), including colorectal cancer, endometrial cancer and various other cancers in heterozygous carriers, one expects to see some of these cancers in the same patients or in the relatives. There is no mention of this in the study, except that they used the revised Bethesda criteria, which include some of these features. In societies with high number of consanguineous marriages, in small genetic isolates, consanguinity may be a marker of increased risk for heterozygosity. Homozygotes and/or compound heterozygotes have been described with very early onset and an NF1-like phenotype,[6] brain tumors, lymphomas and leukemias,[7] and early presentation of colorectal cancers. There are no patients reported in this study with these types of cancer, so it is highly unlikely that this is the case. Consanguinity will usually occur in persons with similar environment and there may be a shared environmental exposure. A number of MSI-H cases are expected by chance (somatic methylation of predominantly MLH1 as a cause of sporadic cancer). That could have been further examined by testing for BRAF mutation,[8] usually present in sporadic tumors caused by methylation of MMR genes, although this was not the aim of this study. It may potentially be a cause of familial late-onset colorectal cancer, but not of HNPCC. It is mentioned that 14 of the 25 MSI-H were over the age of 50, but it is not clear how many of the patients with abnormal MLH1 expression were over 50. High prevalence of MMR gene expression in a study of 154 Saudi patients was reported in this Journal in 2006;[9] it seems that some of the patients are the same (83 patients from KFSH) in both studies. Interestingly in that study[9] 33.8% of the samples showed abnormalities in at least one of the 2 examined MMR genes (protein) tested, while in the present paper[1] this was found in only 11.2% of the cases. There is a big difference between their reported over-expression of p53 (67.3%) and the frequency of the mutations of TP53 (23.8%), which deserves further clarification especially after the claim of lower incidence of TP53 mutations. Could this be due to different mutations from the ones tested, such as exon 9? Or maybe the 2 tests are not testing the same thing. The study of Al-Kuraya et al[9] reported a similar p53 positivity in data reported in the Western literature. How is this discrepancy explained? Approximately 5-20% of most common cancers, usually those of the same type as found in HNPCC, but sporadic in origin, are found to have loss MMR. In this context, colon cancer does not include rectal cancers, the biology of which is distinct. In sporadic colon and endometrial cancers, loss of MMR typically occurs by hypermethylation of the MLH1 promoter, down-regulating its expression. It used to be thought this was a mechanism exclusive to sporadic tumors, but a proportion of HNPCC colon cancers also lose MLH1 via methylation so this is not quite the discriminator that was hoped it might be. Rectal cancers very rarely, if at all lose MMR sporadically, and thus a rectal cancer with MSI or abnormal MMR IHC is excellent evidence of HNPCC. Similarly, colorectal adenomas rarely show MSI outside of HNPCC, so the finding of adenoma with MSI confers a high predictive value of HNPCC. Approximately one third of HNPCC related tumors do not exhibit any abnormality on analysis by IHC even though they have lost MMR function, as manifested by MSI, and this may be due to mutations that functionally

inactivate the MMR protein, but allow its expression as a stable protein with nuclear localization. In the USA, the proportion of colorectal cancer due to HNPCC has been estimated as 1-6%.[10] At 4-6% there is not a statistical significant difference with the finding 8% in the 150 patients of this study.

In the introduction of Bavi et al[1] also mentioned "the study was carried out to see if there was any evidence to support the hypothesis that there were higher number of hereditary tumors in the Kingdom than in the West", "to determine the incidence of HNPCC" and "to examine whether there are any genetic markers which differed from the West to explain the apparent aggression of tumors". None of these objectives was clearly accomplished at the end of the study. Whether the real incidence of HNPCC is higher remains unknown. In our opinion, the number of patients with familial cancer in this study is very limited.

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Reply from the Author

In a letter to the Editor regarding our current article[1] in the Saudi Medical Journal, Bavi et al[1] describe the genomic instability pathways in Saudi colorectal. We would like to clarify number of important issues raised by Drs. Carlos Trujillo and Robert Roger Lebel.

First, we agree with the authors that consanguinity cannot be invoked as a possible explanation of increased incidence of an autosomal dominant condition and where a statement that may suggest that appeared in our paper it was unintended. Rather, genetic variants that operate in an autosomal recessive fashion, such as MYH, are what is meant by our reference to consanguinity in our paper although we do submit that this could have been made more conspicuous. In fact, our group is launching a major project that aims to identify such potential variants and the significance of their contribution to colorectal carcinomas (CRC) in general and MSI in particular. We are perplexed by the authors' reference to familial colorectal cancer X as another example of a familial CRC condition influenced by consanguinity since by definition this recently described form of CRC refers to those families that meet the Amsterdam-I criteria but lack MMR defect in their DNA namely it follows an autosomal dominant pattern of inheritance.[5] Why the authors believe that this autosomal dominant disorder was influenced by consanguinity is unclear to us.

Our study, being pilot in nature, does not have the power needed to address interesting questions that are indeed at the very core of many of the points raised by Drs. Trujillo and Lebel. For example, it was very interesting to calculate the risk of familial CRC that is attributable to MMR defect. Mismatch repair related risk can then be further analyzed in terms of the extent to which MLH1 and MSH2 mutations contribute to such risk. The residual risk left will be an intriguing research question to answer and that is going to be the focus of future work since it could potentially be explained, at least in part, by few high risk alleles that may be operating in a recessive fashion (see above) hence their overrepresentation in our consanguineous population. In this regard, we agree with the Trujillo and Lebel's suggestion that a founder effect could explain the higher risk of MSI among Saudis. This hypothesis will predict that a few germline mutations in MLH1 and MSH2 should account for a large proportion of mutations observed in these genes among Saudis such as show recently in the Finnish population.[11] Again, a lot can be learned when a larger scale study is conducted that also looks at the mutation data on MLH1 and MSH2. The question of founder effect may be less relevant if the significant proportion of the familial risk cannot be attributed to mutations in either of these 2 genes. Regarding the authors concern on other cancers that are associated with Lynch syndrome, although we are in a total agreement with authors that phenotype of Lynch syndrome does indeed include cancers other than CRC. However, we believe that documenting these cancers will add very little information in the way of confirming MMR gene mutations when the expression of MLH1 and MSH2 has already been shown to be absent which, combined with PCR analysis of the 2 microsatellites described in the paper, should leave little doubt that the tumors we classified as MMR related HNPCC are so indeed. The authors are also concerned on other unusual cancer phenotypes that might be associated with consanguineous marriages and potential environmental effect. We further agree that biallelic mutations of MLH1 and MSH2 showed result in the unusual phenotypes described by the authors. However, we would like to reiterate the pilot nature of the paper in question which means that addressing these questions, as intriguing as they are, is beyond its scope. The larger scale study that is underway and that does

involve mutation data on MMR genes will hopefully address whether consanguinity results, as it is predicted to, in increased prevalence of biallelic mutations in these genes. When data from our larger study is available on the residual familial risk of CRC after correcting for all known genetic defects, we speculate on issues such as environmental factors. The authors raised the issue of MLH1 methylation as potential cause of sporadic CRC and its relation to BRAF mutation. We would like to emphasize that in our study of the 19 CRC deficient for MMR proteins by immunohistochemistry, 12 cases were fulfilled using Revised Bethesda guidelines, MLH1 was absent in 4 cases and MSH2 in 8 cases. Loss of MSH2 in the majority of the cases further lends credibility to our postulate that all these cases were HNPCC. Furthermore, we are currently conducting a study to explore the role of BRAF mutation in MMR-deficient Saudi CRC. Interestingly, the incidence rate of BRAF mutations in Saudi CRC is found to be around 2.7% (unpublished data) and that is significantly lower than what was reported in the West.[12] This further supports our hypothesis that HNPCC constitute a higher proportion of Saudi microsatellite unstable CRC. The authors are also concerned on the reported differences in incidence of (a) microsatellite instability by immunohistochemistry and (b) p53 mutation incidence between our current paper[1] and previous study.[9] The difference in MSI incidence can be explained by the following: First, incidence of a biomarker would vary if the cohort size varies and accuracy would increase in a larger cohort. Though incidence of MSI-H in this study was 16.6%, the incidence of MSI-H was 20.2% in a subsequent study where 406 samples were analyzed.[13] Also, refinements and modifications were made in the immunohistochemistry protocol such as the use of a more sensitive IHC detection kit Dako Envision system kit rather than the less sensitive Dako LSAB kit used in our earlier study.

An earlier study has for the first time highlighted the fact that wide spread positive signal in a few cells (heterogeneous staining) does not always correlate with MSS status.[14] This phenomenon of heterogeneous staining in our study might be attributable to inadequate fixation or suboptimal tissue processing protocols, especially since the earlier study was carried out in 2 sets of samples from different laboratories with different processing protocols and some of the King Faisal Specialist Hospital and Research Centre cases were referral cases from peripheral laboratories.[9] Finally, we believe that MSI testing by polymerase chain reaction is the gold standard, which has to be complimented with IHC testing to help in doing methylation followed by mutation analysis on a specific MMR gene. Ethnic differences are known to exist in tumors and these differences may also be accounting for lesser sensitivity as well as specificity of known IHC antibodies in detecting MSI in Saudi colorectal carcinomas.[15] Regarding p53 mutation incidence differences, we would like to clarify that in our study, we discussed the incidence of TP53 mutations by sequencing exons 5-8 and incidence of p53 was 23.8% (27 of 113 colorectal carcinomas) and incidence of p53 overexpression as detected by IHC was 67.3% (95 of 141 colorectal carcinomas). There was a statistically significant correlation between p53 mutation data and p53 IHC results (p=0.0421). Furthermore, we reported this incidence in our submitted manuscript in the results section under heading of "immunohistochemistry".[1] Immunohistochemistry is a major method for investigating p53, based on the observation that mutant p53 protein is frequently stabilized. However, there are a number of problems that lead to false-negative and false-positive results.[16-18] Wild-type p53 is stabilized by physiological stimuli such as hypoxia, specific oncogenic stresses, or DNA damage resulting from the presence of free radicals from tumor-associated macrophages or following therapy, leading to positive staining in the absence of mutation.[19,20] On the other hand, immunohistochemistry is negative in tumors in which p53 is inactivated by loss of both alleles or by null mutations (which predict poor outcome[21] and not all mutations stabilize the protein).[18] Also there are conflicting reports on the prognostic significance of p53 expression by IHC in colorectal carcinomas. Though some reports have shown overexpression of p53 by IHC to be associated with worse outcome in colorectal carcinomas,[22,23] others have failed to show prognostic value of p53 expression in colorectal carcinomas.[24,25] Thus we chose to present the mutation analysis of p53 by direct sequencing analysis, which is the gold standard and can detect the heterozygous mutation when as little as 5% of the genomic DNA contains the mutated gene.[26] However, we have also mentioned our p53 IHC results and the fact that IHC p53 expression correlated significantly with p53 mutation data. Furthermore, we analyzed p53 mutations in a large cohort of 386 colorectal carcinomas and p53 mutations were detected in 33.7% (130 of the 386) colorectal carcinomas.[13] p53 mutations showed a trend towards older age (p=0.0728), histology subtype of adenocarcinomas (p=0.0809), larger tumor size (p=0.0590) and were significantly associated with lymph node metastasis (p=0.0464). An inverse correlation was seen between MSI status and TP53 mutations, which was statistically significant (p=0.0043). Thus TP53 mutation incidence in Saudi colorectal carcinomas ranged from 23.8-33.7%, which is lower than the range of 40-60% reported in the West

and this was mentioned in the discussion. Finally we agree with the authors that our current study could not determine the exact incidence of HNPCC in Saudi population and that is due to inherent limitations of genomic DNA material and lack of proper family history, however we believe that the findings in this paper are novel and will for sure pave the way for future studies.

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