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ARTICLE

Two Germline Alterations in Mismatch Repair Genes Found in a HNPCC Patient with Poor Family History

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The Bethesda guidelines may offer more useful criteria in patients' selection for germline mismatch repair gene mutation analysis than guidelines merely based on family background. An early onset double primary colorectal cancer patient with poor family history with MSI-H status was investigated for MLH1 promoter methylation, expression of the MLH1 and MSH2 gene by immunohistochemistry and mutations in the MLH1 and MSH2 genes. The index patient carried two germline alterations, the p.Val716Met in MLH1 and the c.2210+1G>C in MSH2 genes, and both tumors failed to express MLH1 and MSH2 proteins. After subsequent analysis of the whole family of the index patient, the p.Val716Met variant can be defined as a rare polymorphism with the possible contribution of pathogenicity to tumor formation and c.2210+1G>C as a true pathogenic mutation causing an out-of-frame deletion of exon 13. (Pathology Oncology Research Vol 12, No 4, 228–233)

Keywords: Bethesda criteria, hereditary nonpolyposis colorectal cancer, hMLH1, hMSH2, microsatellite instability, immunohistochemistry

Introduction

HNPCC is the most common form of inherited colorectal cancer syndromes associated with an increased lifetime risk for mainly colorectal or endometrial cancer. Colorectal cancer has an annual incidence of 70 cases/100,000 inhabitants in Hungary. Hereditary cancer accounts for 5 to 10 percent of all colorectal cancer cases; two thirds of the familial cases are diagnosed as hereditary nonpolyposis colorectal cancer (HNPCC).[1] Beside HNPCC families that fulfill the Amsterdam criteria there are patients with a single early onset colorectal cancer or with multiple synchronous or metachronous primary tumors without any family history. These features could also suggest HNPCC syndrome. HNPCC is most frequently caused by germline mutations in the MLH1 and MSH2 genes, but rarely other mismatch repair genes are involved.[2,3] Microsatellite instability (MSI) is a hallmark of mismatch repair deficiency that may result not only from mutations in the mismatch repair genes but also from epigenetic inactivation of MLH1. Hypermethylation of the MSH2 promoter region in colorectal cancer has not been observed.[1] Immunohistochemistry (IHC) can predict which mismatch repair gene is expected to harbor a mutation.

The Bethesda guidelines were introduced to identify patients with colorectal cancer who should be tested for microsatellite instability.[4] In patients fulfilling the Bethesda criteria, microsatellite instability testing is an important tool to identify hereditary colorectal cancer, especially in patients who do not meet the Amsterdam or extended Amsterdam criteria.[5] All patients with colorectal cancer under the age of 50 with high microsatellite instability, even in the absence of a significant family history, should undergo molecular analysis of the two mismatch repair genes.[6]

Here we report on a 25-year-old male patient with synchronous colorectal cancer without a family history who was identified among the patients being screened for the necessity of mutation analysis in the mismatch repair

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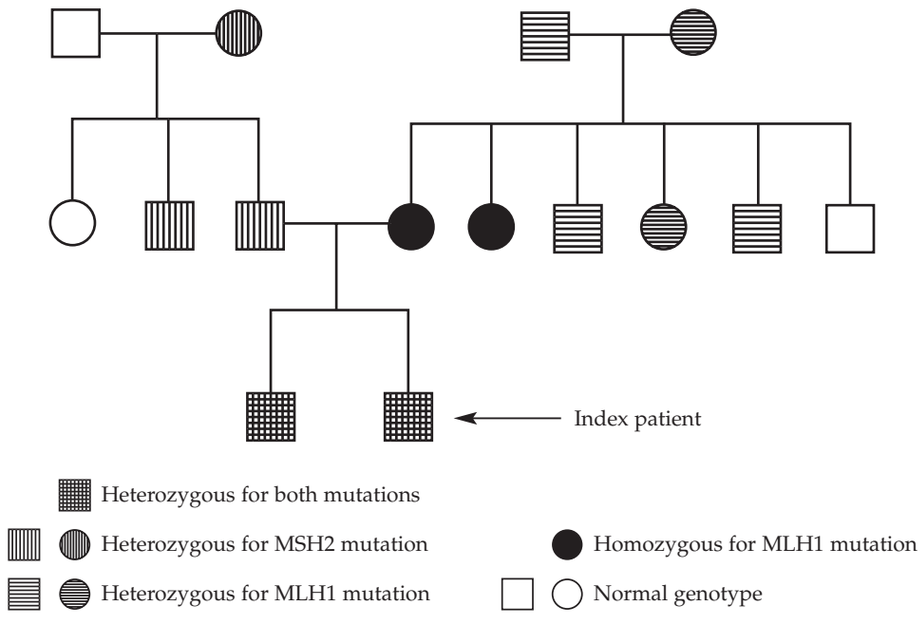

Figure 1. *Pedigree of the examined family with indication of the germline alterations. The index patient developed two synchronous colorectal cancers by the age of 25. His brother has already developed colorectal adenoma. The paternal grandfather carries none of the germline mutations although he developed sporadic colorectal cancer over the age of 80.*

genes. In accordance with the Bethesda guidelines, the patient was tested for MSI and hMLH1 promoter methylation as well as for mutations in the MLH1 and MSH2 genes. The family members of the index patient were subsequently analyzed for the mutations found.

Materials and Methods

Patients and DNA samples

A patient with multiple synchronous colorectal carcinoma was selected on the basis of the Bethesda criteria for mutation analysis at the 1st Department of Surgery, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary. The patient did not meet the Amsterdam or the Amsterdam II criteria. He was 25 years old at the diagnosis of both carcinomas. One of the tumors was detected in the cecum (Grade 3; stage: T3, N0, M0; Dukes' B2), the other was located in the rectum (Grade 3; stage: T2, N1, M0; Dukes' C1). Blood samples were collected from the family members. The parents, the 28-year-old brother, the uncles and aunts in their 50s or 60s had no history of cancer, however, the paternal grandfather developed colon cancer over the age of 80 (*Figure 1*). DNA from both the paraffin-embedded tumor tissues and the corresponding blood sample of the patient as well as from blood samples of the family members was extracted after proteinase K (Sigma, St. Louis, MO) digestion according to protocol of the High Pure PCR Template Purification kit (Roche Diagnostics GmbH, Mannheim, Germany).

Microsatellite analysis

DNA samples isolated from the two tumors and the corresponding blood were used for testing MSI. Two mononucleotide repeat markers (BAT25 and BAT26) and three dinucleotide repeat markers (D2S123, D5S346 and D17S250) were studied according to the international reference panel recommendations[7] using the HNPCC Microsatellite Instability Test (Roche Diagnostics GmbH). The MSI status was assessed according to the consensus of the National Cancer Institute workshop on Microsatellite Instability for Colorectal Cancer Detection.[7] High level instability (MSI-H) was diagnosed when more than 30% of the examined markers exhibited new alleles in the tumor tissue, whereas low level instability (MSI-L) was established when less than 30% of the markers carried instability. Where no instability was observed in any of the markers examined it was classified as microsatellite stable (MSS) phenotype (*Figure 2*).

hMLH1 promoter methylation assay

The assay on the region between –316 and –435, proximal to the transcriptional start site of the promoter, was performed with methylation-sensitive and non-sensitive enzymes and inner controls.[8] The assay for the distal region between –662 and –575 was performed with quantitative real-time PCR by comparing the methylation status to an unmethylated sperm control and a fully methylated artificial control.[9]

PCR, heteroduplex analysis (HAD) and single strand conformation polymorphism (SSCP)

All exons of the hMLH1 and hMSH2 genes were analyzed in the blood sample of the patient. Primers and cycling conditions used were published earlier by Beck[10] and Yanagisawa.[11] After denaturation the PCR products for HDA and SSCP were subjected to electrophoresis on MDE gel (Cambrex Bio Science Rockland Inc., Rockland, ME) according to the manufacturers' instruction, and visualized by silver staining.

Sequencing reactions

Direct DNA sequencing was performed on the purified PCR products showing altered migration patterns by HDA or SSCP. The analysis was carried out using BigDye terminator cycle sequencing kit v.3.1 (Applied Biosystems, Foster City, CA) and the products were run on an ABI-PRISM 310 genetic analyzer (Applied Biosystems).

Immunohistochemistry

Immunohistochemical analysis was performed on paraffin-embedded 5-micrometer-thick tissue sections. Sections were dewaxed, rehydrated, and microwaved for 20 minutes in citrate buffer (pH 6.4). Non-specific binding was blocked by bovine serum albumin in PBS. Sections were then incubated with the following primary monoclonal antibodies for 1 hour at room temperature: mouse anti-human MLH1 (G168-728) and mouse anti-human MSH2 (G219-1129) (both from Cell Marque, Hotsprings, AR) (*Figure 3*). The negative control was processed the same way but with the omission of the primary antibodies. Antibody binding was detected by a secondary antibody and biotin-streptavidine detection kit (LSAB, Dako, Glostrup, Denmark) using VIP chromogen (Vector Laboratories, Inc., Burlingame, CA). Negativity was declared in the absence of any nuclear signal in tumor cells. Nuclei were counterstained with methyl green (Dako).

Results

A patient suffering from colorectal carcinoma, with little family history of cancer was selected for mutation analysis. He satisfied three Bethesda criteria: multiplex synchronous colorectal cancer, cancer diagnosed at the age of <45 years, and a first-degree relative with colorectal adenoma diagnosed at the age of <40 years.

Detection of microsatellite instability

Both tumors of the patient had MSI-H phenotype (an instability ratio of 80 and 80 percent, respectively) (*Figure 2*). Both tumors showed instability for the following markers: BAT25, BAT26, D2S123 and D17S250, and only the D5S346 marker was stable. The altered alleles of the mononucleotid repeats (BAT25, BAT26) had different sizes in the two tumor tissues.

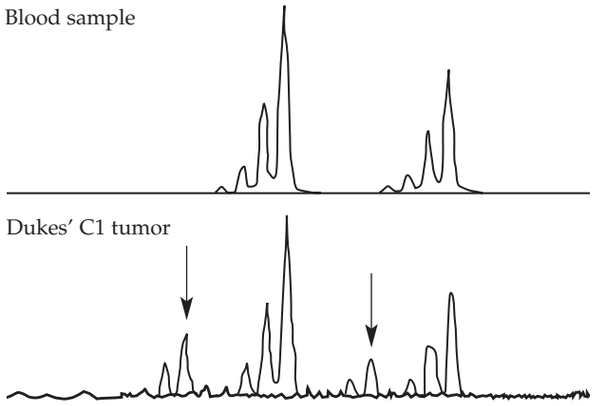

Figure 2. *Microsatellite analysis of the Dukes′ C1 tumor and the blood samples showed microsatellite instability high phenotype at the D2S123 marker. Arrows indicate new alleles appearing in the tumor sample.*

Immunohistochemistry

By immunohistochemistry, we failed to detect the MSH2 or MLH1 proteins in the nuclei of the tumor cells in either investigated cancers (*Figure 3*), which may be due to the lack of expression of these two mismatch repair genes.

Methylation of the hMLH1 promoter region

Promoter hypermethylation of the hMLH1 gene was not present in either the proximal or the distal region of the gene promoter in either of the tumors of the patient. The ratio of hypermethylation in the distal region detected by quantitative real-time PCR was 5 percent for the Dukes' B2 tumor and 12 percent for the Dukes' C1 tumor, both of which thus belonging to the low frequency category.

MSH2 and MLH1 mutations

Two MMR gene alterations were identified in both the cecal and rectal tumors of the patient. In subsequent analysis, both of the mutations were also detected in his blood sample, proving that these alterations were of germline origin. One alteration was detected in the hMLH1 gene in exon 19, a valine to methionine amino acid change at codon 716 (p.Val716Met), the other alteration was located in the hMSH2 gene in intron 13, a G>C change at 2210+1 nucleotide (c.2210+1G>C). The

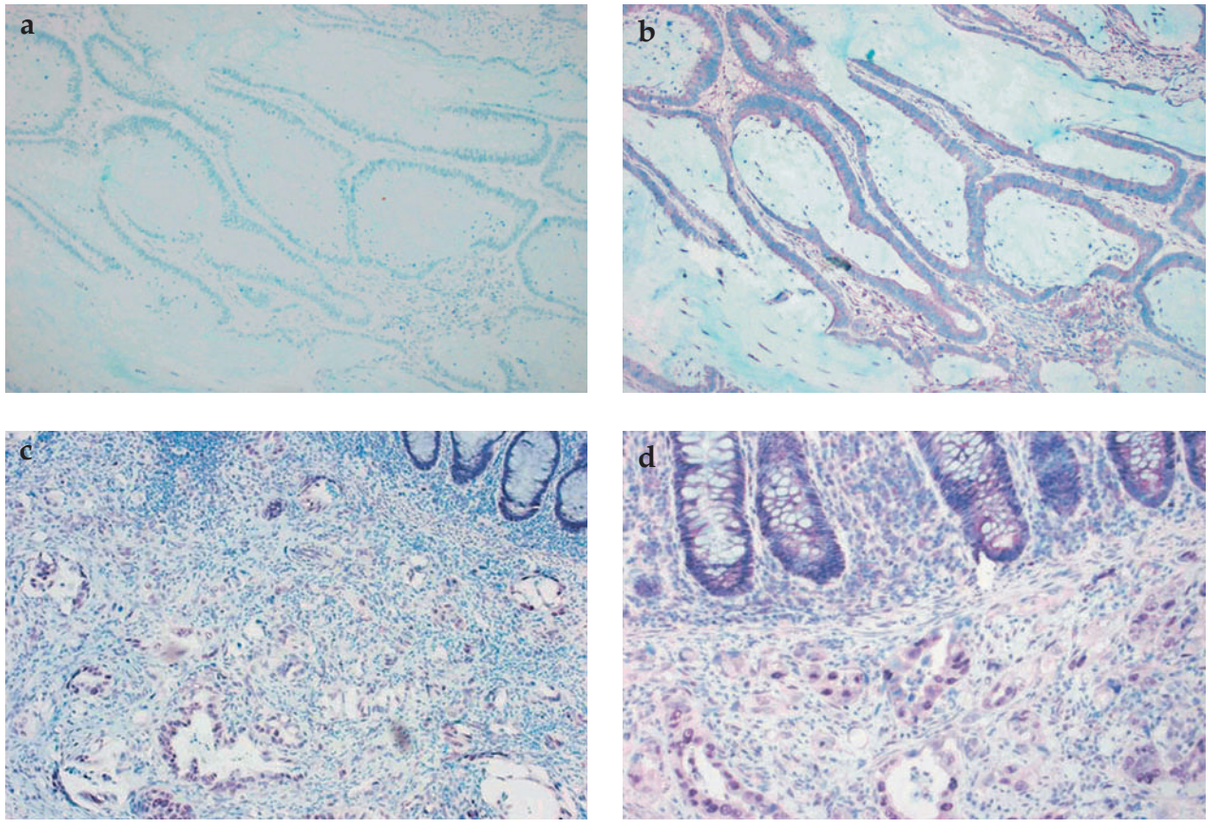

Figure 3. *Immunohistochemical analysis of the G3 Dukes' B2 tumor showing complete loss of expression of MLH1* ***(a)*** *and MSH2* ***(b)*** *with malignant control samples expressing MLH1* ***(c)*** *or MSH2* ***(d)*** *protein. Original magnification: x100*

patient was heterozygous for both of the alterations. His parents, brother, uncles and aunts and grandparents were all screened for these two alterations (*Figure 1*). The father, his brother, the father's brother and the paternal grandmother were heterozygous for the c.2210+1G>C change in MSH2. Interestingly, the patient's mother and one of her sisters were homozygous, while two of her brothers, one of her sisters and the maternal grandparents were heterozygous for the p.Val716Met in MLH1. The patient's brother carried the same two alterations as the index patient, and his paternal grandfather had none of these mutations.

Discussion

The patient studied here fulfills three criteria of the Bethesda guidelines and does not fulfill the Amsterdam or the extended Amsterdam criteria. Among these, the Bethesda guidelines represent the least strict criteria, many patients with diverse clinical features meeting them. Since the emergence of molecular diagnosis of HNPCC, however, it has become evident that the Amsterdam or extended Amsterdam criteria do not cover all HNPCC patients and that a subset of these patients may be identified among those meeting the Bethesda criteria only. To identify patients with HNPCC syndrome, microsatellite instability testing is a useful tool that can be followed by mismatch repair gene analysis if necessary.[5]

In our patient the MSI status in both tumor tissues was very high, with four of the five analyzed markers showing instability, which suggests that the patient has HNPCC syndrome. The different sizes of the extra alleles showed that the tumors were two independent synchronously developed primary tumors.

Immunohistochemistry cannot replace MSI analysis as a prescreening method because of its lower sensitivity.[12] However, it can be used to predict which gene is expected to harbor the mutation.[13] The MSI results of the patient correlated with the IHC findings: both of the tumors with abnormal protein expression showed MSI. The loss of expression of two MMR genes detected by IHC and the high MSI status suggest that this patient has alterations in both MMR genes, and thus has HNPCC syndrome.

Epigenetic DNA modification such as MLH1 promoter methylation was not observed in the two tumors or the corresponding blood sample. Nevertheless, published results

have shown no association between clinical features and methylation status in HNPCC tumors with microsatellite instability.[5,14]

In the family studied the index patient had two germline alterations, one of them was p.Val716Met in the hMLH1 gene and the other was a c.2210+1G>C change in the hMSH2 gene. The p.Val716Met was first identified by Hutter et al in a HNPCC family[15] together with another germline mutation, and this variant was defined as a putative disease causing mutation. Cederquist et al[16] found this mutation in a patient with double primary metachronous tumors (endometrial at the age of 50 and colorectal at the age of 56) together with another pathogenic mutation, and defined p.Val716Met sequence alteration as an unclassified variant. In our family the mother of the index patient and her sister also carry this mutation in homozygous form, and her two brothers, one sister and the maternal grandparents carry it in heterozygous form, and have developed no cancer. The amino acid at this position is evolutionarily not conserved,[17] and the variant had been identified in approximately 1 percent of normal controls by Myriad Genetic Laboratories (A.M. Deffenbaugh and L.A. Burbidge, personal communication). The allele frequency of this amino acid change was also analyzed by Cederquist et al and also found to be 1 percent in healthy individuals.[16] Therefore this variant could rather be classified as a rare polymorphism. The fact that the patient's mother and her sister with homozygous p.Val716Met alteration have not developed cancer also confirms this conception. This variant may not be a sufficient explanation for the loss of expression of the MLH1 gene. Considering that the analysis was limited to the promoter region and the coding sequence, the patient may harbor a somatic regulatory mutation.

The second alteration was the c.2210+1G>C change in MSH2, which was described earlier as a pathogenic mutation by Kurzawski et al[18] in a Polish HNPCC family. This mutation causes an out-of-frame deletion of exon 13, as the nucleotide change affects a splice site at the exon-intron boundary. We also found this mutation in heterozygous form in the father of the patient, in the father's brother, in the paternal grandmother and in the index patient's brother who have not developed cancer.

The paternal grandfather developed colorectal cancer over the age of 80, however, he carried none of these mutations. Both his age and the fact he is a non-carrier suggest that his colorectal cancer was a sporadic disease.

The 28-year-old brother has the same variants as the index patient, therefore, his clinical examination was recommended. As a result, an adenoma in the colon was detected that can be an early step in the progression to cancer and thus requires follow-up.

Our results confirm that with the use of the Bethesda guidelines as criteria to perform MSI testing, several patients with HNPCC syndrome can be identified who would be missed by the application of the Amsterdam or the extended Amsterdam criteria. The Amsterdam criteria exclude patients without family history, however, the presence of certain clinical features might be sufficient to identify further patients with MSI-H status. Among the alterations found in the index patient the p.Val716Met variant described earlier as a disease-associated mutation should rather be considered a polymorphism that may contribute to the effect of the other mutation. Its presence without another mutation did not cause early onset colorectal cancer either in heterozygous or in homozygous form. On the other hand, the c.2210+1G>C alteration seems to be a true pathogenic mutation causing an out-of-frame deletion by affecting a splice site at the boundary of exon 13. We hypothesize that the adverse effect of the c.2210+1G>C mutation is enhanced by the presence of the p.Val716Met polymorphism thus causing earlier cancer development.

Acknowledgement

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References

1. *Cunningham JM, Christensen ER, Tester DJ, et al:* Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. Cancer Res 58: 3455-3460, 1998.
2. *Wang Q, Lasset C, Desseigne F, et al:* Prevalence of germline mutations of hMLH1, hMLS2, hPMS1, hPMS2, and hMSH6 genes in French kindreds with nonpolyposis colorectal cancer. Hum Genet 105: 79-85, 1999.
3. *Syngal S, Fox EA, Eng C, et al:* Sensitivity and specificity of clinical criteria for hereditary non-polyposis colorectal cancer associated mutations in MSH2 and MLH1. J Med Genet 37: 641-645, 2000.
4. *Rodriguez-Bigas MA, Boland CR, Hamilton SR, et al:* A National Institute workshop on hereditary nonpolyposis colorectal cancer syndrome: meeting highlights and Bethesda guidelines. J Natl Cancer Inst 89: 1758-1762, 1997.
5. *Raedle J, Trojan J, Brieger A, et al:* Bethesda guidelines: Relation to microsatellite instability and MLH1 promoter methylation in patients with colorectal cancer. Ann Intern Med 135: 566-576, 2001.
6. *Montera M, Resta N, Simone C, et al:* Mutational germline analysis of hMSh2 and hMLH1 genes in early onset colorectal cancer patients. J Med Genet 37: E7, 2000.
7. *Boland CR, Thibodeau SN, Hamilton SR, et al:* A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 58: 5248-5257, 1998.
8. *Kámory E, Kolacsek O, Ottó S, et al:* hMLH1 and hMSH2 somatic inactivation mechanisms in sporadic colorectal cancer patients. Pathol Oncol Res 9: 236-241, 2003.
9. *Eads CA, Lord RV, Wickramasinghe K, et al:* Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res 61: 3410-3418, 2001.

10. *Beck NE, Tomlinson IPM, Homfray T, et al:* Use of SSCP analysis to identify germline mutations in HNPCC families fulfilling the Amsterdam criteria. Hum Genet 99: 219-224, 1997.
11. *Yanagisawa Y, Akiyama Y, Iida S, et al:* Methylation of the hMLH1 promoter in familial gastric cancer with microsatellite instability. Int J Cancer 85: 50-53, 2000.
12. *Wahlberg SS, Schmeits J, Thomas G, et al:* Evaluation of microsatellite instability and immunohistochemistry for the prediction of germ-line MSH2 and MLH1 mutations in hereditary nonpolyposis colon cancer families. Cancer Res 62: 3485-3492, 2002.
13. *Hendriks Y, Franken P, Dierssen JW, et al:* Conventional and tissue microarray immunohistochemical expression analysis of mismatch repair in hereditary colorectal tumors. Am J Pathol 162: 469-477, 2003.
14. *Raut CP, Pawlik TM, Rodriguez-Bigas MA:* Clinicopathologic features in colorectal cancer patients with microsatellite instability. Mut Res. 568:275-282, 2004.
15. *Hutter P, Couturier A, Membrez V, et al*: Excess of hMLH1 germline mutations in Swiss families with hereditary non-polyposis colorectal cancer. Int J Cancer 78: 80-84, 1998.
16. *Cederquist K, Emanuelsson M, Goransson I, et al:* Mutation analysis of the MLH1, MSH2 and MSH6 genes in patients with double primary cancers of the colorectum and the endometrium: a population-based study in northern Sweden. Int J Cancer 109: 370-376, 2004.
17. http://prodes.toulouse.inra.fr
18. *Kurzawski G, Suchy J, Kladny J, et al:* Germline MSH2 and MLH1 mutational spectrum in HNPCC families from Poland and the Baltic States. J Med Genet 39: E65, 2002.

Corrections of Pathology Oncology Research

In the 12/3 issue in the article Thanaa El A HELAL et al: „Human papilloma virus and p53 expression in bladder cancer in Egypt: Relationship to schistosomiasis and clinicopathological factors": *the published Fig. 3 is incorrect. The ovum is on the right side and not the left.*