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K-RAS GENE MUTATION AS AN EARLY PROGNOSTIC MARKER OF COLON CANCER

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Due to increased colorectal cancer incidence there is a necessity of seeking new both prognostic and prediction factors that will allow to evolve new diagnostic tests. K-ras gene seems to be such a factor and its mutations are considered to be an early marker of progression of colorectal cancer.
The aim of the study was to find a correlation between K-ras gene mutation in patients with diagnosed colorectal cancer and selected clinical parameters.
Material and methods. A total of 104 patients (41 women and 63 men) with diagnosed colorectal cancer were included in this study. The average age of male group was 68.3 and in female group – 65.9. Samples were taken from paraffine blocks with tissue from diagnosed patients and K-ras gene mutation were identified. Afterwards the statistical analysis was made seeking the correlation betweenK-ras gene mutation incidence and clinical TNM staging system, tumour localisation, histological type, sex, age.
Results. K-ras gene mutations were detected in 20.1% of all colorectal cancers. Significantly higher rate of K-ras gene mutations were diagnosed among patients classified at stage I (40%), stage IIC (50%) and stage IV (50%) according to the TNM classification.
Conclusions. The results of our study are compatible with other studies and indicate the correlation between K-ras gene mutation and colorectal cancer incidence. Identification of K-ras gene mutation may complement other diagnostic methods at early stage of colorectal cancer.
Key words: K-ras gene mutation, colorectal cancer

Colorectal cancer (CRC) is one of the most commonly diagnosed malignant cancers in developed countries, including Poland. The worldwide incidence rate of this cancer is estimated at 1.2 mln. Highest incidence rates are observed in Japan, North America, some regions of Europe, New Zealand and Australia, low incidence rates – in Africa and South-East Asia (1). The most common type is adenocarcinoma, which accounts for 98% of all colorectal cancer types (2). The increasing incidence rate of this cancer causes a significant oncologic problem in the modern world.

Despite the introduction of latest diagnostic and therapeutic approaches, treatment outcomes remain unsatisfactory and the 5-year survival rates in specialist oncology centres rarely exceed 60% (3).

The aim of the study was to search for a relationship between a K-ras mutation in patients with known colorectal cancer and certain clinical parameters.

MATERIAL AND METHODS

The study enrolled 104 patients treated at the 2nd Department and Chair of General and Oncological Surgery of the University Teaching Hospital in Wrocław. CRC location and stage was determined using the TNM classification. The histological grade was determined

according to a three-stage classification adopted by the World Health Organisation in 2000 (G1-G3).

The study material was collected from paraffin-embedded blocks of tissue from patients with confirmed colorectal cancer. The study cancer tissue was fixed using a standard procedure, in 10% formalin for approx. 48 hours, then the collected specimens were dehydrated with a graded alcohol series, cleared with xylene and embedded in low melt point paraffin. Biopsy specimens collected from analyzed cases in the study group were rediagnosed in specimens routinely stained with hematoxylin and eosin.

The selected patient group with study material was used for assessment of K-ras mutations. The study used Real-Time PCR (Polymerase Chain Reaction), with Light Cycler 480 Real-Time PCR System from Roche.

RESULTS

All patients in the study group comprising of 104 subjects were diagnosed with CRC. The study group comprised 41 women (39.4%) and 63 men (60.6%). The average age for men was 68.3 years and for women – 65.9 years. (tab. 1).

Tumor location was as follows: caecum -13 cases (12.8%), ascending colon – 6 (5.9%), hepatic flexure – 3 (2.9%), transverse colon – 3 (2.9%), splenic flexure – 5 (4.9%), descending colon – 5 (4.9%), sigmoid colon – 43 (42.2%), rectosigmoid flexure – 4 (3.9%), rectum – 20 (19.6%). Moreover 2 cases of synchronous cancer have been diagnosed: 1 case of synchronous cancer of the transverse colon and splenic flexure and 1 case of synchronous cancer of the caecum and transverse colon.

Cancer staging according to TNM classification showed stage I cancer – 10 (9.6%) cases; stage IIA – 38 (36.5%) cases; stage IIC – 4 cases (3.8%); stage IIIA 6 (5.8%) cases; stage IIIB 35 (33.7%) cases; stage IIIC 3 (2.9%) cases and stage IVA 8 (7.7%) cases.

Distribution of cancers by histological grade was as follows: G1 – 9 (8.8%) cases; G2- 86 (84.3%) cases and G3 – 7 (6.9%) cases.

K-ras mutations were identified in 23 (20.1%) of 104 patients with colorectal cancer.

When cases were classified according to cancer location in colorectal cancer, K-ras mutation was identified in 9 (69.2%) cancers located in the caecum; in 3 cancers located in the ascending colon (50%); one K-ras mutation in cancer located in the transverse colon (33.3%), the splenic flexure (20.0%) and descending colon (20%). Eight cases of K-ras mutations have been reported in cancers located in the sigmoid colon (17.8%). In all 20 cases of rectal cancer and 4 cases of rectosigmoid flexure cancers K-ras mutation was not identified. Similarly, no K-ras mutations have been identified in cancers located in the hepatic flexure. There were also no K-ras mutations identified in both cases of synchronous cancer.

According to the cancer staging system, a high proportion of K-ras mutations was identified in stage I (4 cases; 40%), stage IIC (2 cases: 50%) and stage IV (4 cases; 50%). No K-ras mutations were identified in stage IIIC cancers and there was a small proportion of K-ras mutations in stage IIIB cancers (7 cases; 20%) (tab. 2).

DISCUSSION

Long-standing studies have demonstrated that colon carcinogenesis is a long-term and multistage process, the integral part of which are mutations of genes involved in the initiation of tumor development (4).

CRC is therefore a genetically mediated disease and develops as a result of mutation accumulation in the genome of existing,

Table 1. Characteristics of the study group

Sex	Size (a total of 104 patients)	Mean age	Number of mutations
Women	41 (39,4%)	65,9	12 (29,3%)
Men	63 (60,6%)	68,3	11 (17,4%)
Total	104	67,1	23 (22,1%)

Table 2. Characteristics of K-ras mutations frequency according to predefined criteria

Location	Number of cases (n=102, cases of synchronous cancer were excluded)	Number of mutations
Caecum	13 (12,8%)	9 (69,2%)
Ascending colon	6 (5,9%)	3 (50%)
Hepatic flexure	3 (2,9%)	0 (0%)
Transverse colon	3 (2,9%)	1 (33,3%)
Splenic flexure	5 (4,9%)	1 (20%)
Descending colon	5 (4,9%)	1 (20%)
Sigmoid colon	43 (42,2%)	8 (18,6%)
Rectosigmoid flexure	4 (3,9%)	0 (0%)
Rectum	20 (19,6%)	0 (0%)
Histological grade	Number of cases (n=102, cases of synchronous cancer were excluded)	Number of mutations
G1	9 (8,8%)	3 (33,3%)
G2	86 (84,3)	18(20,9%)
G3	7 (6,9%)	2 (28,6%)
Histological grade TNM	Number of cases n=104	Number of mutations
I	10 (9,6%)	4 (40%)
IIA	38 (36,5%)	5 (13,6%)
IIC	4 (3,8%)	2 (50%)
IIIA	6 (5,8%)	1 (16,7%)
IIIB	35 (33,7%)	7 (20%)
IIIC	3 (2,9%)	0 (0%)
IVA	8(7,7%)	4 (50%)

healthy cells. These mutations lead to phenotypic malignancy presented by the cancer lesion, including development of mutated cells and their proliferation. Uncontrolled growth of aggressive cells results in infiltration (invasion) and occupation of areas otherwise normally occupied by other cells (metastasis).

K-ras gene is located on the short arm of chromosome 12 at position 12.1, that encodes a 21-kDa protein (5). The gene has two alternative exons 4 – 4A and 4B. Exons are involved in gene expression in two different proteins K-ras 4A and K-ras 4B (6). Isoform K-ras 4B is prevalent in the colon and accounts for approx. 99% of expression. Oncogenic K-ras mutations lead to impairment of GTPase function and result in active protein-GTP nucleotide complex (7).

Polymerase chain reaction is an effective technique used in molecular biology that allows for prompt detection, amplification, i.e. copying and identification of trace amounts of nucleic acids – even single copies of the studied sequence (8). However the need to differentiate between the unmutated type and various mutant allele, i.e. precise genotype evaluation, requires the use of PCR modification analyses.

Real-time detection of mutations in PCR tests uses two methods ARMS and Scorpions (9).

Each mutation test labelled with FAM contains one Scorpions primer and one ARMS primer for differentiation between wild type DNA and mutated DNA detected with real-time PCR.

Allele-specific amplification or mutation-specific amplification is performed using ARMS technology. Taq DNA Polymerase is particularly effective in differentiating between aligned and unaligned nucleotides at the 3' end of the PCR primer. When the primer is fully aligned, this allows for full performance amplification. Scorpions probes are used for the detection of amplification. Scorpions probes are bifunctional molecules containing a primer covalently linked to the probe (10).

During PCR Real-Time the amount of fluorescence generated in the course of PCR is recorded and the results are reported as threshold cycle value (CT) which is the cycle number (C) at which the fluorescence crosses the threshold (T).

Samples are classified as mutation positive status if their CT is lower than 1% of the test

CT value. Above this value, the sample may contain less than 1% of mutation or be mutation negative (11).

K-ras mutations are monoallelic, point, usually somatic mutations, which develop at an early stage of CRC carcinogenesis. Vast majority of mutations are changes in sense codons 12 (approx. 82%) and 13 (approx. 17%) in exon 1 (12).

Studies in various populations worldwide demonstrated that the most common mutation in codons 12 and 13 is the transition from guanine to adenine and guanine to thymine transversion (7). Point mutations in codons 12 and 13 at the first and second triplet result in changed amino acid sequence of the protein. Glycine to aspartate substitution and glycine to valine substitution are most commonly observed (13).

Codon 12 GGT and codon 13 GGC are responsible for incorporation of glycine into K-ras protein formed during translation (a biogenic amino-acid with no side chain). Glycine at position 12 is situated in a tight loop to which the GAP protein binds. Any mutation in this position will result in the incorporation of a side-chain amino-acid, and thus preventing, in terms of spatial architecture, the transition of the protein to inactive status, i.e. GDP-bound form in the presence of GAP. K-ras mutations result therefore in a continuous activation status of this protein. This results in an uncontrolled cell growth and cell division (14).

K-ras mutations, particularly glycine to valine substitution (mutation in G12V codon) may be an independent risk factor for overall survival in patients with colorectal cancer.

Moreover, the literature data suggests that mutations which activate the RAS signalling pathway are also important prognostic factors in patients treated with monoclonal anti-EGF (epidermal growth factor) receptor antibodies, when development of mutation results in unresponsiveness to treatment (15).

The primary aim of the study was to evaluate a relationship between K-ras mutations in patients with colorectal cancer and certain clinical factors. The incidence rate of K-ras mutations in the study group was 20.1%. Other studies reported the incidence rate of this mutation at 15-50% (13, 16, 17). Such a wide range of incidence rates may result from application of various study methodologies (different methods for collection and preparation of material).

We have observed that the incidence rate of K-ras mutations is lower in men (17.4%) compared to women (29.3%). Similar conclusions were made by other authors, who emphasized that particularly lower incidence rate is observed in men aged below 40. These papers presented a thesis that this is related to female sex hormones, which have an effect on the carcinogenesis process (17).

In our study material no K-ras mutations were identified in any of the 20 cases of CRC; also no K-ras mutations were identified in 2 cases of synchronous carcinoma.

We have observed a relationship between a proportion of K-ras mutations and the histological grade. The highest incidence rate for K-ras mutations has been identified in G1 stage study material (33,3%), compared to G2 stage (20.9%) and G3 stage (28.6%).

High detection rate of K-ras mutations was observed in TNM stage IV colorectal cancer (50%) which in our opinion may indicate an accelerated course of the disease and higher disease aggressiveness, especially that no K-ras mutations were identified in stage IIIC and a small proportion (20%) was identified in stage IIIB. Our observations are supported in literature, where authors report a significantly lower survival of patients with an identified K-ras mutation (18). A relatively high identification rate of mutations in stage I CRC (40%) may indicate an incidental early detection of disease of potentially high level of aggressiveness.

CONCLUSIONS

Results from our studies confirm the reports of other authors and indicate a correlation between K-ras mutation and the development of colorectal cancer. The determination of K-ras mutation development may be supplementary to existing diagnostic methods in early-stage CRC, and particularly may be an additional parameter for evaluation of degree of malignancy in CRC.

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