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Detection of K-ras Genes Expression and Mutations Genes in Thyroid Cancer Among Sudanese Patients

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Abstract

Background: The molecular pathways and tumorigenicity are remaining the only key for developing a new gene therapy approach to achieve efficient and successful antitumor efficacy with minimum normal tissue toxicity. The prevalence rate of K-ras mutations in thyroid carcinoma makes it a viable genetic marker as well as a useful prognostic tool, and it has a crucial role in malignant transformation and tumor progression.

Objective: To detect K-ras genes expression and mutations genes in thyroid cancer among Sudanese patients.

Materials and methods: In this cross-sectional study, a total of 104 formalin-fixed, paraffin-embedded tissue blocks previously diagnosed as thyroid tumors were collected from seven governmental hospitals in Khartoum State (Sudan). It included 43 follicular carcinoma cases, 43 papillary carcinoma cases, five follicular variant papillary carcinoma cases, seven medullary carcinoma cases, four anaplastic carcinoma cases, and two metaplasia cases. K-ras antibody immunohistochemistry technique (Gen Tex. North America) was performed on tissue microarray slides to measure the protein expression of K-ras in all malignant cases. Also, block folds of 10µm thickness sections were collected from same tissues to perform polymerase chain reaction (PCR) in Eppendrof tubes and then underwent RFLP in the site of mutation. The data were analyzed using the Statistical Package for Social Sciences, version 20.

Results: High expression 47.1% (49/104) was revealed in benign tumors; while K-ras protein expression in malignant neoplasms was 31.7% (33/104). This difference showed a significant association (p = 0.017). On the other hand, there was no association between the histopathological diagnosis subtypes and K-ras protein expression (p-value = 0.161). Expression of KRAS codon 12 gene mutation detected in benign tumors was 27/30 (90%); and that detected in malignant tumours was 67% (67/100). This difference showed a significant association (p = 0.000). Also, there was an insignificant association of K-ras codon 13gene mutation with the histopathological diagnosis (p = 0.307).
Furthermore, there was a high significant association between the histopathological diagnosis

subtypes and K-ras codon12 gene mutation ((p = 0.000). K-ras protein expression detected in different tumour subtypes was: follicular carcinoma 48.8% (43/88), papillary carcinoma 32.9% (29/88) follicular variant subtype 5.6% (5/88), medullary carcinoma 7.9% (7/88), and anaplastic carcinoma 4.5% (4/88). There was no significant association between K-ras codon 12gene mutation and K-ras protein expression (p = 0.494); and no significant association between K-ras codon13gene mutation and K-ras protein expression (p = 0.596).

Conclusion: K-ras protein expression was significantly associated with the histological diagnosis, but insignificantly associated with the tumour subtypes. K-ras codon12 gene mutation was significantly associated with the histological diagnosis and malignancy subtypes. No significant association was detected between K-ras codon 13 gene mutation and K-ras protein expression.

Keywords: Thyroid cancer, K-ras genes expression, Mutation genes, Tumour subtypes.

Introduction

K-ras is the name given to a family of related genes that encode a class of 21 KD membrane-bound proteins that bind guanine nucleotides and have intrinsic GTPase activity. The K-ras gene inherent the catalytic activity which encodes a188 amino acid protein. The post-translation modification of this protein facilitates its localization to the cell membrane. K-ras mutations comprise 86% of all K-ras mutations. Mutations in K-ras occur with the greatest frequency in all human cancers (21.6%), a better understanding of this gene, as well as its interactions with other genes and mutations, has recently revealed its potential prognostic and predictive roles in tumor aggressiveness and patient outcomes[1].

Point mutations in the K-ras gene are the most common mutations. Mutations in codon 12 and 13 lead to an increased affinity for GTP. Mutations in codon 61 lead to inactivation of the K-ras protein's innate GTPase function. overall, these mutations result in the constitutive activation of the K-ras protein and thus the activation of the downstream signaling pathways regulates a critical step in thyroid tumor genesis. Thyroid carcinoma has been associated with mutations in all three isoforms of the K-ras genes . K-ras mutations occur with variable frequency in all types of thyroid follicular-derived tumors. K-ras point mutations are most common in follicular thyroid carcinoma (FTC), occurring in 40-50% of tumors, as well as in poorly differentiated and Anaplastic thyroid carcinoma (PDC and ATC). It is more infrequent in PTCs, occurring in about 10%of tumors. The prevalence of K-ras mutations in thyroid carcinoma makes it a viable genetic marker as well as a useful prognostic tool, given that, studies suggest it may increase the potential for malignant transformation and tumor progression[2].

The pathogenesis of thyroid carcinoma with K-ras mutation occurs as an early event in follicular adenoma and may increase the potential for malignant transformation. Other studies suggested that K-ras may predispose well-differentiated thyroid cancer (WDTC) to subsequent de-differentiation into poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC). Oncogenic modifications in K-ras have been hypothesized to be one of the first steps in TC development because of the presence in well-differentiated TCs (WDTCs) and poorly differentiated PDTC or anaplastic ATC and medullary MTC. The most frequent mutation was

K-ras (24.3%), followed by NRAS (8.4%) and HRAS (4.7%). PDTC and ATC were the histological subtypes with the greater incidence of K-ras mutations (55.2% and 51.7%), respectively[3].

Papillary (PTC) is the most common form of thyroid cancer, accounting for about 75-80% of thyroid malignancies. FTC cases account for about 15% of all thyroid cancer cases. This type of thyroid cancer is more aggressive than PTC and tends to spread through the bloodstream to other parts of the body (vascular invasion is characteristic of follicular carcinoma), and therefore distant metastasis is more common. ATC is the least common (accounting for 5%-15% of primary malignant thyroid neoplasm) and the most deadly type of thyroid cancer. MTC is a neuroendocrine malignancy that secretes excessive amounts of CT, frequently metastasizes and has limited treatment. MTC accounts for 3 to 10% of all thyroid cancer cases but bears significant mortality[4].

K-ras oncogenes and PTC, it became clear that the K-ras mutations are predominantly related to poorly differentiated thyroid carcinomas and anaplastic thyroid cancers than PTC which suggests the role of K-ras is more inclined to the progression rather than the initiation of tumors. That Knowledge of K-ras mutation status has potential diagnostic value and can aid in the selection of targeted therapies that the presence of K-ras mutation in intermediate cytology changes the decision of the initial surgical management. Although identification of K-ras mutation in the fine needle aspiration biopsy (FNAB) specimen is not 100% predictive of cancer, it is certainly highly suggestive of either follicular carcinoma TC or follicular variant papillary thyroid carcinoma (VPTC)[5].

Materials and methods

In this cross-sectional study, a total of 104 embedded paraffin blocks previously diagnosed as thyroid tumors were collected from seven governmental hospitals in Khartoum State (Sudan). The study was approved by the Scientific Research Committee of Alzaiem Alazhari University, Khartoum North, Sudan, and the Ethical Board of the Ministry of Health (Sudan). Data confidentially was maintained, and the information collected from all specimens had not been used for any purpose other than this study. Permission to investigate the specimens was granted from the directors of the seven governmental hospitals in Khartoum State from which specimens were collected. Informed consent from patients has been waived by the ethical committees, since patients' identity was anonymized, and only laboratory numbers were used.

The study investigated 43 follicular carcinomas, 43 papillary carcinomas, five follicular variant papillary carcinomas, seven medullary carcinomas, four anaplastic carcinomas, and two metaplasia cases. Immunohistochemistry technique was performed on tissue microarray slides using K-ras antibody (GenTex, North America) to measure the protein expression of K-ras genes in the malignant tumours. Also, from the same blocks, tissues (4fold of 10µm thickness) were collected for polymerase chain reaction (PCR) test in Eppendrof tubes and then underwent RFLP in the site of mutation.

Tissue microarray (TMA): From each hospital block hematoxylin and Eosin sections were prepared to determine microscopically the cellular malignant part. Then from the donor blocks and recipient blocks 2 cores (3mm in diameter) were taken from each case. The cores

were arrayed manually (about 40 cores in each block). Again hematoxylin and eosin staining was used for all sections of the recipient blocks. Then sections underwent immunohistochemistry run in positively charged slides. Then using IHC kits (H_2O_2) blocker, enhancer, horse radish peroxidase (One step), and DAB chromogen. Mayer hematoxylin was used as a counterstain.
Immunohistochemistry procedure: Applying the procedure of detection system kit (Biogenx, California, USA), the immunohistochemistry (IHC) procedure was started the step of antigen retrieval (1:20 dilution) in a water bath at 95°C for 30 min, and cooled for 10 min. Then endogenous peroxidase block hydrogen peroxide was added for 10 min and washed for 2min in buffer (1:50 dilution).
Then the primary K-ras anti-body was applied (1:10 dilution) for 10 min. Then the enhancer was applied for 20 min, washed with the buffer for 2min, and horse radish polymer was added for 15 min, then washed with the buffer for 2 min and chromogen DAB substrate (1drop-1 ml) was added. The colour was noted first with the naked eye and under the microscope for 5 min. The slides were then counterstained with Mayer's hematoxylin for 1min. The slides were dehydrated in ethanol, cleared in xylene, and then mounted in DPX[6].
After staining the stained slides were examined under the microscope for positivity and intensity determination of the protein expression of K-ras genes (noted in the cytoplasmic membrane). Lastly, the results of recipient blocks were mapped.
DNA extraction: 4 sections of 10 µ size were cut with the rotary microtome (Lieca, USA) under sterile conditions and collected in 1.5 ml labeled Eppendrof tubes for DNA extraction and polymerase chain reaction amplification of exon 2 (codon 12 and 13) of the K-ras genes. Genomic DNA was extracted from 10 µm sections of formalin-fixed and paraffin-embedded (FFPE)-xylene/ethanol. Sections were incubated overnight proteinase K, and subsequently DNA was purified by using the QI Amp DNA FFPE tissue kit (Qiagen, Hilden, Germany). Then the DNA concentration was measured.
Gene-specific PCR primers were designed for primarily DNA extraction from FFPE tissues and to flank mutational hotspots regions in K-ras exons 2. PCR products were checked by a run of electrophoresis on 2% agarose gels. DNA extraction was performed using the Qiagen kits of the formalin-fixed, paraffin-embedded sections. Primers were designed according to bioinformatics programs (NCBI). The primers blast applied were from Macrogen Company (Korea):
* Forward: 5′- GACTGAATATAAACTTGTGGTAGTTGGACCT-3′.
* Reverse: 5′- CTATTGTTGGATCATATTCGTCC-3′
* Forward: 5′- GACTGAATATAAACTTGTGGTAGTTGGACCT-3′.
* Reverse:5′-CTATTGTTGGATCATATTCGTCC-3′

PCR protocol: Amplification protocol for K-ras codon 12 was performed first by initial denaturation at 94°C for 2min, denaturation at 94°C for 20 seconds, annealing at 53°C for 10 sec, extension at 72°C for 30 seconds and final extension at 72°C for 5 min. (40 cycles).
The amplification protocol for K-ras codon 13 was performed first by initial denaturation at 94°C for 5 min, denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1min. and final extension at 72°C for 5 min. (40 cycles).
Protocol of K-ras codon 12 using Maxime PCR pre-mix kit (i-taq- iNtRON, Korea) was performed by preparing serial dilutions of the primer. The most suitable dilution of K-ras codon

12 and 0.5 ml was selected for each forward and reverse primers ((Humanizing Genomic Macrogen, Korea):
* K-ras codon 12 forward: 5′- GACTGAATATAAACTTGTGGTAGTTGGACCT-3′.
* K-ras codon 12 reverse: 5′-CTATTGTTGGATCATATTCGTCC-3′.
After serial dilutions the most suitable dilution of the KRAS codon 13 primer selected was 1 ml for each forward and reverse primers as follows:
* K-ras codon 13 forward: 5′- ATTTTTATTATAAGGCCTGCTG-3′.
* K-ras codon 13 reverse: 5′- TCAAGGCACTCTTGCCTAGG-3′.
RFLP Protocol for K-ras codon 12 exon 2: After PCR amplification of K-ras codon 12, the length of band yield was 107 base pairs (bp). It was digested by BstnI, Mval restriction enzyme (Thermo Scientific, EU, Lithuania). The wild-type of K-ras exon-2 allele was cleaved into two fragments of 77 and 30 bp, while the mutant type remained intact at 107 bp.
RFLP Protocol for K-ras codon 13 exon 2: After PCR amplification of K-ras codon 13, the length of band yield was 83 bp. It was digested by BsuR1, Hai111, restriction enzyme (Thermo Scientific, EU, Lithuania). The wild-type K-ras codon 13 exon-2 allele was cleaved into three fragments of 48, 20, and 5 bp; while the mutant type remained intact (83 bp).
Restriction Enzyme Protocol: This protocol was performed by adding 3.8 µl distilled water, 1µl 10x buffer, 0.2 µl restriction enzyme, and 5µg PCR product. Mixture was then incubated overnight at 37°C in a heat block where digestion took place. After that mixture was inactivated at 80°C for 20 min, then the restricted band was inoculated in the gel electrophoreses system.
Gel electrophoresis: The 10x buffer was prepared by adding boric acid (13.7g) to tris base (27g) and EDTA (1.86g). Then the mixture was completed to 250 ml with double distilled water. Two concentrations of the agarose preparation were used at 2% and 3% agarose (Intron, Korea).
2 g % (2g dissolved in 100 ml1x buffer) for PCR band detection and 3 g % (3g in 100 ml 1xbuffer) for digested band were used after enzyme restriction detection. The Ladder (Sizer TM-50 DNA marker. Korea) (100bp and50bp) were used to determine the band length[7].
Statistical analysis: Frequencies, mean values, standard deviations (SD) and ranges were used as descriptive statistics. The relationship between the positivity of the K-ras mutation and the histopathological grading was tested by means of the Mantel-Haenszel chi-square test for linear association. The statistical analysis was performed by running the SPSS/PC+ statistical package version 20, on a personal computer. A two-tailed P of 0.05 was chosen as the cut-off for detecting statistically significant values.

Results

The association between histopathological diagnosis and K-ras protein expression using the immunohistochemical marker showed a significant association (p = 0.017). The high expression revealed 47.1% (49/104) benign tumors, and 31.7% (33/104) malignant neoplasms (Table 1).
As shown in Table (2), the association of the histopathological diagnosis with gene mutations of KRAS codon12 was highly significant (p = 0.000).
However, the association of the histopathological diagnosis with gene mutations of K-ras codon13 was found insignificant (p = 0.307). Also, the K-ras codon 12 gene mutations was highly detected in benign tumors

Table(1): Association of the histopathological diagnosis with the immunohistochemical (IHC) K-ras expression

IHC K-ras expression	Benign tumours	Malignant tumours	p - value
Negative	55 (52.8%)	71 (68.2%)	0.017
Positive	49 (47.1%)	33 (31.7%	
Total	104 (100%)	104 (100%)	

(27/90%); while it was (67/67%) in malignant tumours. There was a significant association between the histopathological diagnosis of the tumour subtypes (follicular carcinoma, papillary carcinoma, follicular variant subtype, medullary carcinoma, and anaplastic carcinoma) and K-ras codon12 gene mutations (p = 0.000).

In K-ras codon 12 gene mutations, a high frequency rate of follicular carcinoma (48.8%) was detected; while the frequency rates of other tumour subtypes were (32.9%), (5.6%), (7.9%), and (4.5%) for papillary carcinoma, follicular variant carcinoma, medullary carcinoma, and anaplastic carcinoma (Fig. (1).

Table(2): Association of histopathological diagnosis with K-ras codon12 and K-ras codon13 gene mutation

IHC **K-ras** expression		Benign tumours	Malignant tumours	p - value
K-ras codon12	Wild type	3 (10%)	33 (33%)	0.000
	Mutant type	27 (90%)	67 (67%)	
	Total	30 (100%)	100 (100%)	
K-ras codon 13	Wild type	28 (93.3%)	94 (90.3%)	0.307
	Mutant type	2 (6.7%)	10 (9.7%)	
	Total	30 (100%)	104 (100%)	

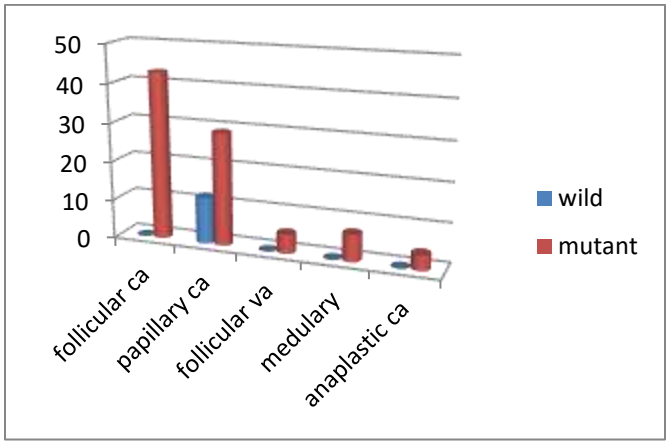

Fig. (1): Association between the histopathological diagnosis of tumour subtypes and the genes mutations of K-ras codon 12

Discussion

In this study, KRAS genes expression marker showed a significant association with the histopathological diagnosis (p = 0.017). The highest expression was detected in benign tumors (47.1% - 49/104). In malignant neoplasms, this marker frequency rate was (31.7% - 33/104). There was no significant association between the **K-ras** expression marker and the malignant tumour subtypes (p = 0.161).

Berna and his colleagues[8] (2014) reported that the prevalence rate of **K-ras** genes mutations was approximately 20-40%. They disagree with the findings that **K-ras** mutations were detected in follicular adenomas and follicular variant papillary thyroid carcinoma. Also, Deligiorgi and his co-workers (2018) [9] disagreed with the finding that **K-ras** expression was revealed in 47.4% of papillary carcinomas and the predominant **K-ras** had expressed PTC histotype and emerged the classical PTC comprising 66.7% of PTC.

On the other hand, this study showed a highly (90% - 27/30) significant association between **K-ras** codon 12 and benign tumors (p = 0.000); while in malignant tumours **K-ras** genes expression was (67% - 67/100). Anelli and his co-authors (2014)[10] reported a similar result where **K-ras** gene mutations were observed in both benign and malignant thyroid neoplasms. These mutations could be found in 40-50% of FTC cases, in 5-20% of PTC cases, in 20-40% of poorly differentiated and anaplastic cases, as well as in approximately 30% of follicular adenomas. **K-ras** positive follicular adenoma may be a precursor of both follicular cancer and follicular variant of papillary carcinoma.

Furthermore, Berna and his colleagues[16] had agreed with the findings of this study where **K-ras** mutations were detected in follicular adenomas and in follicular variant papillary thyroid carcinoma.

Also, Ricarte-Filho and his co-workers (2009)[11] reported that **K-ras** mutations were more common than BRAF mutations (12% vs. 44%), i.e. p = 0.002. They estimated the mutation frequency rate of **K-ras** as (17.4%).

Gina and his co-authors (2013)[12] reported an overall frequency rates of **K-ras** mutations up to 48% in benign follicular adenomas, 57% in FTC, and 21% in PTC.

In the present context, KRAS codon 12 gene mutation had the highest frequency rate in follicular carcinoma (48.8% -43/88); and it was (32.9% - 29/88) in papillary carcinoma. Many studies supported the findings of our study. Raphael Rubin and his colleagues (2012)[13] reported that **K-ras** proto-oncogenes were mutated in less than 10% in PTC and 20-45% in FTC.

Mingzhao Xing (2013)[14] reported that the mutations in **K-ras** genes frequency rate was up to 45 % in follicular thyroid cancer (FTC), 30-45 % in follicular variant papillary thyroid cancer (FVPTC), 20-40 % in poorly differentiated thyroid cancer (PDTC), 10-20 % in anaplastic thyroid cancer, rarely in classical papillary thyroid cancer (PTC), and also in benign tumours such as follicular thyroid adenoma.

Medici and his co-authors (2016)[15] reported that **K-ras** mutations had an important role in the clinical behavior of thyroid tumors and assist in the management of thyroid nodules and thyroid cancer. **K-ras** mutations alone had a diagnostic value, it is a component of the genetic marker panel that has high negative predictive values and can thus help to rule out thyroid malignancy. When **K-ras** mutation were positive and cytologically benign, the thyroid nodules can be

managed without surgery but followed for the long periods. If **K-ras** mutations appeared with other genetic alterations, the tumor should be treated more aggressively in appropriate clinical settings and proper therapy regime.

Conclusion: K-ras protein expression was significantly associated with the histological diagnosis, but insignificantly associated with the tumour subtypes. **K-ras** codon12 gene mutation was significantly associated with the histological diagnosis and malignancy subtypes. No significant association was detected between KRAS codon 13 gene mutation and **K-ras** protein expression.

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