

Original Research Article

Assessment of Kirsten sarcoma viral gene mutations in non-neoplastic and neoplastic nodular lesions of thyroid in Pakistan and identification of subsets of classical papillary carcinoma associated with KRAS mutations

ABSTRACT

Objectives: To assess KRAS mutations in non-neoplastic and neoplastic nodular lesions of thyroid and to identify subsets of classical papillary carcinoma associated with KRAS mutations. The incidence of KRAS mutations in thyroid lesion in Pakistan has not been evaluated.

Methods: The cross sectional study was conducted at Basic Medical Sciences Institute, Jinnah Postgraduate Medical Center, Karachi from 2011 to 2014. 70 cases including 6 multinodular goiters, 10 hyperplastic nodules, 10 follicular adenomas, 7 WDUMPs, 4 follicular carcinomas, 22 classical papillary carcinomas, 11 follicular variant of papillary carcinomas were subjected to standard PCR to detect KRAS mutations located at codon 12 exon 1

Results: KRAS mutations located at codon 12 exon 1 were found in 02(2.87%) cases of multinodular goiter, 05(7.14%) hyperplastic nodules, 02 (2.87%) follicular adenoma , 03(4.2%) (WDUMP). Among malignant lesions follicular carcinoma showed KRAS positivity 03(4.2%), classical papillary carcinoma 08(11.42%) and follicular variant of papillary carcinoma 07(10%).

Conclusion: Our data suggests strong presence of KRAS mutations in malignant tumors supporting the presence of KRAS mutations in our population particularly follicular variant of papillary carcinoma. Follicular variant is a discrete variant of papillary carcinoma having strong association with KRAS mutations.

INTRODUCTION:

Thyroid neoplasm represents most common endocrine malignancy with rising incidence [1]. It constitutes 90% of all endocrine cancers. Thyroid lesions most commonly present as thyroid nodules in 4-7% of population out of which 5-6.5% are malignant [2]. The thyroid cancer incidence has tripled, the reason could be increasing use of modern detection techniques and diagnostic criteria changes [3].

Worldwide thyroid cancer is the 16th most common cancer in 2012 [4]. In USA estimated prevalence of thyroid cancer in 2016 was 64,300 [5]. According to Shaukat Khanum cancer hospital and Research registry in 2013 thyroid carcinoma is the 10th most common malignancy in females with Papillary carcinoma being the most common histological type [6].

Possible risk factors for thyroid cancer are iodine deficiency or excess, radiation exposure and organo chlorinated pollutants [3, 7-9]. Histologically thyroid neoplasms are classified as Papillary, Follicular, Medullary and Anaplastic tumors.

There has been substantial increase in understanding of molecular genetics of thyroid cancer over the past couple of years [10]. Various mutations are involved in thyroid neoplasms such as *BRAF*, *RAS*, *RET/PTC* and *PAX/PPAR* gamma rearrangements. *RAS* has been implicated as fundamental physiological and pathological process in controlling cell growth, differentiation and survival. *RAS* comprises of 150 distinct cellular members [11].

RAS accounts for second most common genetic alteration. *RAS* gene has 3 isoforms *NRAS*, *HRAS*, *KRAS* [1]. *NRAS* mutation at codon 61 is by far most common [10]. Thyroid neoplasms are unique as all of 3 isoforms of *RAS* are reported in thyroid neoplasms [12]. *KRAS* is the 6th most common genetic mutation in all cancers but its frequency varies among thyroid cancer and is reported by Catalogue of somatic mutations in cancer (Cosmic) as *NRAS* 6%, *HRAS* 4%, *KRAS* 2 to 3% respectively [3, 13]. The mutations in *KRAS* gene are mostly located at positions 12 and 13 in exon 1 and less in codon 61 close to the GTP binding site [14].

Studies have demonstrated that thyroid cancers bearing *KRAS* mutations have strong impact on tumor dedifferentiation, aggressiveness and distant metastasis [15]. There is a great discrepancy regarding overall frequency of *KRAS* mutations in follicular patterned lesions, some studies have reported incidence of *KRAS* mutations in colloid nodule 1%, adenoma 6%

follicular carcinoma 1.6% and papillary carcinoma 2.7% [8]. The reason for this discrepancy may be the small sample sized studies and difference in techniques [1]. It was observed that *KRAS* mutations were seen more frequently in malignant than benign thyroid lesions [8]. The incidence of *KRAS* mutations in Pakistan has not been evaluated. This study was designed to detect *KRAS* mutations in neoplastic as well as non-neoplastic nodular thyroid lesions particularly in classical papillary carcinoma and its morphological variants as *KRAS* mutations are associated with tumor aggressiveness and poor outcome [3].

MATERIALS AND METHODS:

Study approval was obtained from ethical committee of Basic Medical Sciences Institute, Jinnah Postgraduate Medical Center, Karachi with reference number (NO.F.1-2/2015/BMSI-E-COMT/022/JPMC) on 11 March 2015 and procedure was in accordance of Declaration of Helsinki. Properly fixed paraffin embedded surgical pathological specimens of patients who underwent thyroidectomy during 5 years (2011-2014) were retrieved from previous records. 70 cases were selected; these included 6 multinodular goiters, 10 hyperplastic nodules, 10 follicular adenomas, 7 WDUMPs, 4 follicular carcinomas, 22 classical papillary carcinomas, 11 follicular variant of papillary carcinomas. Toxic multinodular goiter, metastatic carcinoma and non follicular thyroid neoplasm were excluded from study.

The DNA extraction from tissue was carried by using Epicenter Kit (cat#MCD 85201) and the protocol was followed accordingly. In 1.5 ml appendroff cup 50 mg of tissues was taken from paraffin block and 600 µl T and C lysis solution was added along with diluted 1 µl of 50µg/µl Protinase K to all the sample tubes. Mix and incubate overnight at 37°C then all the tubes were vortex and 20 µl of MPC (Protein Precipitation Reagent) was added to 600µl of lysed sample and was vortexed vigorously. Samples were centrifuged at 13000 rpm for 10 minutes and supernatant was obtained. The supernatant was transferred into another tube in which 500 µl of isopropanol was already added. All the tubes were inverted 30-40 times

83 slowly to recover the DNA from supernant. The tubes were centrifuged for 10 minutes at
84 13000 rpm. The supernatant was discarded without disturbing the pellet. Then 500 µl 100%
85 ethanol was added into pellet and was centrifuged for 7 minutes at 13000 rpm to remove all
86 the isopropanol. The supernant was discarded and tubes were placed inverted slowly without
87 disturbing the pellet .The pellet was air dried and the pellet was resuspended in 35 µl of TBE
88 buffer. All the samples were safed at -80°C till PCR was performed

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90 Genotyping by standard PCR was performed in a tube containing 200 µl of reaction mixture
91 made up of the following components: 20 pmol of each primers (forward and reverse),
92 500µm of four deoxynucleotides, 2.5 U of Taq polymerase (Promega), 10 X PCR buffer
93 containing and 1.5mM MgCl₂. The thermal cycler (Master Gradient PCR system, Eppendorf
94 AG, Germany) was programmed to first incubate the sample for 5 minutes for 95°C followed
95 by 45 cycles consisting of 94°C for 45 seconds, 55°C for 50 seconds and 72°C for 1 minute,
96 30 seconds respectively with final extension for 8 minutes at 72°C. The PCR amplified
97 products were identified by gel electrophoresis.

98 At the end of the run the gel was under transilluminator gel doc and bands of amplified
99 product were evaluated. The amplified product was compared with 100-bp DNA ladder
100 (GibcoBRL, Life Technologies) and mutation was detected and compared with control
101 positive and negative cases.

102 Point mutations located at positions 12 in exon 1 were dectected. PCR primers were
103 used for amplification. *KRAS* positive control was used by repeating the positive cases which
104 were detected during this project and for negative control water was used.

105 **DATA ANALYSIS:**

106 Data was entered on a data sheet. Percentage of *KRAS* positive mutations in
107 neoplastic and non neoplastic lesions were calculated.

RESULTS:

KRAS mutations located at codon 12 exon 1 were found in 02(2.87%) cases of multinodular goiter while 05(7.14%) hyperplastic nodules were positive for *KRAS*. In benign neoplastic lesions of thyroid 02 (2.87%) follicular adenoma showed *KRAS* mutations. Among borderline lesions (WDUMP) 03(4.2%) cases were *KRAS* positive. Among malignant lesions most of follicular carcinoma showed *KRAS* positivity 03(4.2%). Amongst 22 classical papillary carcinoma only 08(11.42%) showed *KRAS* mutations whereas out of 11 cases of follicular variant of papillary carcinoma 07(10%) showed *KRAS* mutations.

DISCUSSION:

The thyroid nodular lesions have marked heterogeneity varying from multinodular goiters, adenomas to carcinomas. The histopathological diagnosis of thyroid lesions has been a significant problem and has been a subject of debate in the literature [16].

Our study showed mutated *KRAS* at codon 12 on exon 1, showing conformity other studies also reported similar findings. A higher proportion of positive *KRAS* was found in neoplastic lesion 23 (32.69%) followed by non-neoplastic 7(10.1%). Our findings are comparable to international studies who also reported that *KRAS* mutations are more frequent in neoplastic lesions than in non-neoplastic lesions [8].

***KRAS* in Benign lesions:**

Amongst the benign lesions in this study *KRAS* mutations were present in 2.87% multinodular goiter. The frequency of *KRAS* mutations in multinodular goiter varies in different studies ranging from 0% to 10%, 2.17% and 1% respectively [8, 17]. Overall *RAS* mutations i.e. *NRAS*, *KRAS* and *HRAS* are reported as 0%, 6.5% and 7%. Our study observed *KRAS* mutations in a small percentage of hyperplastic nodules i.e 7.14%. The overall reported

RAS mutations in hyperplastic nodules are 5.6% [17]. These benign lesions may later transform into neoplastic lesions and further support the hypothesis of Nikiforov (2011) that nodules harbouring *KRAS* mutations may be categorized as follicular adenoma as they are probable true neoplasms [10].

Regarding benign neoplastic lesions, *KRAS* mutations were found in 2.87% cases of follicular adenoma. Our findings are comparable to Vasko et al. (2003) reporting exon 1 *KRAS* mutation in 0.6% of follicular adenoma while most of their cases showed *NRAS* mutations i.e 14% [8]. Our results could not be compared to other studies because studies regarding *KRAS* mutations in follicular adenomas are limited.

***KRAS* in Malignant lesions:**

Follicular carcinoma in this study shows 4.2% *KRAS* mutation. Our results are slightly higher as compared to Vasko et al. (2003) who reported exon 1 *KRAS* mutations in 1.6% cases of follicular carcinoma while most of their cases showed *NRAS* mutations [8]. Nikiforov et al. (2003) and Schulten et al. (2013) on the contrary reported that none of their cases of follicular carcinoma showed *KRAS* mutation [17, 18]. Nikiforov (2011) has reported overall *RAS* mutations in 40% to 50% of conventional type follicular carcinoma, however no specific *RAS* isoform was mentioned in his study [10]. Garcia-Rostan et al. (2003) have reported correlation between *RAS* mutations and metastatic behavior of tumor [15]. Our figures could be related to tumor aggressiveness in *KRAS* positive follicular carcinoma. Further studies are required on extensive sample size for detection of *KRAS* mutations in follicular carcinoma.

For follicular variant of papillary thyroid carcinoma this study showed mutated *KRAS* in codon 12 on exon 1 in 10% cases. Our results are higher in comparison with Rivera et al. (2010) who reported *KRAS* mutations in a small number of cases 1/28 (3.57%) and Park et al. (2013) reports 22.9% [19, 20]. Zhu et al. (2003) on the contrary reported that none of his

cases of FVPTC showed *KRAS* mutation [21]. According to Park et al. (2003) *KRAS* mutations were positive in most papillary carcinoma with follicular growth pattern suggesting phenotypic difference between classical papillary carcinoma and its follicular variant [20]. According to Rivera et al. (2010) follicular variant of papillary carcinoma has a molecular profile close to follicular adenoma/carcinoma[19]. Nikiforov (2011) has postulated that *RAS* positive papillary carcinoma with follicular histology show frequent encapsulation, less obvious nuclear features of papillary carcinoma and lower chance of lymphatic spread[10]. The present study however, emphasizes the association of FVPTC with *KRAS* mutations in our population.

Classical papillary carcinoma in this study showed 11.4% positivity for *KRAS* mutations. These figures are higher compared to international study reporting 2.7%. A higher percentage of 54.5% was reported by Schulten et al. (2013).[17] Overall *RAS* i.e. *KRAS*, *NRAS* & *HRAS* mutations are reported by Esapa et al. (1999) 8%, Basolo et al. (2000) 10% and Zhu et al. (2003) 17% respectively.[21],[22, 23]A higher result was expected in our study but according to Myers et al. (2012) and Vasko et al. (2003) discrepancy may be due to small sample size, epidemiological differences and as well as due to the fact that though *KRAS* mutations are often present in higher numbers of tumors, but exists as mutant fraction that are too small to be detectable by direct sequencing that can be missed by Real time PCR and can only be detected by ACB-PCR (Allele –specific competitive blocker PCR)[3, 8].

Present study showed *KRAS* mutations (4.2%) in well differentiated tumors of uncertain malignant potential (WDUMPT) According to Hofman et al. (2009) none of his cases showed *KRAS* mutations while *NRAS* was present in 1/31(3.22%) cases[24]. Probably these figures are related to likelihood of a possible malignant transformation in these lesions. The information regarding *KRAS* mutation in WDTUMP was limited in literature so comparisons were difficult.

The presence of oncogenic *KRAS* is a marker of tumor aggressiveness and poor outcome and may guide future course of treatment.

CONCLUSION:

Majority of malignant tumors showed *KRAS* mutations supporting the presence of *KRAS* mutations in our population. Follicular variant is a discrete variant of papillary carcinoma having strong association with *KRAS* mutations whereas Classical papillary carcinoma did not showed significant *KRAS* mutations as mutated *KRAS* are often present as mutant fraction which can be missed by DNA sequencing techniques, even by quantitative real time PCR and can only be detected by more sensitive techniques such as ACB-PCR (Allele –specific competitive blocker PCR).

Benign lesions harbouring *KRAS* mutations may serve as malignant precursors. This study showed that *KRAS* mutation were found in variable percentages of various thyroid proliferative lesions ranging from multinodular goiters, hyperplastic nodules, follicular adenomas, WDUMPs, classical papillary carcinomas, and follicular variant of papillary carcinomas.

RECOMMENDATIONS:

1. More studies on other centers of Pakistan are needed to know the actual prevalence of *KRAS*.
2. Malignant tumors particularly classical papillary carcinoma should be evaluated for *KRAS* mutations as tumors carrying *KRAS* mutations are more aggressive and unlikely to benefit from targeted therapies such as Epidermal growth factor receptor (EGFR) as *KRAS* leads to its downregulation.

Limitation: Due to fianancial constraints standard PCR was done and only 70 samples were tested.

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TABLE

DISTRIBUTION OF SELECTED NEOPLASTIC AND NON-NEOPLASTIC

LESIONS OF THYROID ACCORDING TO KRAS MUTATIONS

(n=70)

Morphological type	KRAS mutations +ve (%)	KRAS mutations –ve (%)
Neoplastic		
FA	2 (2.87%)	8 (11.42%)
WDTUMP	3 (4.2%)	4 (5.71%)
FC	3 (4.2%)	1 (1.4%)
PTC	8 (11.42%)	14 (20%)
FVPTC	7 (10%)	4 (5.71%)
Total	23(32.69%)	31(44.24%)
Non-neoplastic		
MNG	2 (2.87%)	4 (5.71%)
HN	5 (7.14%)	5 (7.14%)
Total	7(10.1%)	9(12.85%)

FA=Follicular adenoma, WDUMP=well differentiated tumor of uncertain potential, PT=Papillary thyroid carcinoma,
FVPTC=follicular variant of papillary carcinoma, MN=multinodular goiter, FA=follicular adenoma