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Original Article

Evaluation of Diagnostic and Prognostic Relevance of Single Nucleotide Polymorphism at let-7 micro RNA-binding site of KRAS3 /UTR in Oral Squamous Cell Carcinoma

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ABSTRACT

Objective: Asingle nucleotide polymorphism at Let 7 miRNA binding site in 3/ UTR of KRAS, creates a variant allele KRAS -LCS6 observed to impart poor prognosis in oral cancers. Objective of this study was to explore diagnostic and prognostic relevance of *KRAS-LCS6 variant allele in* oral squamous cell cancer.

Methods: Study was conducted on a total of 100 cases and 100 age and sex matched healthy controls after taking informed written consent. Proposal was approved by Institutional Review Board of Ziauddin University. KRAS–LCS6 SNP genotyping was done via Taqman allelic discrimination assay using allele specific primers and probes ordered from macrogen on real time PCR instrument.

Results: Mean age at presentation was 45.12 ± 12.42 for cases and 44.22 ±12.2 for controls p>0.05. Male to female ratio among cases was 2.2:1. Majority (70%) of cases had a moderate differentiation, and nodal metastasis (96 %). KRAS-LCS 6 variant allele resulting from a single nucleotide polymorphism at Let 7 miRNA binding was observed only in 4 patients and none of the healthy controls. All 4 subjects had a heterozygous genotype (TG) with homozygous genotype (GG) observed in none of the cases or controls.

Conclusion: The variant allele does not exist in this cohort of patients and hence KRAS LCS6 SNP genotyping is not an ideal marker for early detection or predicting disease outcome in our patients with oral cancer.

Keywords: OSCC, Oral cancer, KRAS-LCS6, Let7 miRNA binding site, Single nucleotide polymorphism

Introduction

miRNAs and their target gene deregulations have emerged as a new catalogue of molecular markers in oral cancer. Literature search opens up a huge plethora of miRNAs and their target genes deregulations involved in oral cancer. miRNAs bind to the 3/UTR (un translated region) of target gene messenger RNA causing translational repression or degradation depending on degree of complementarity.[1]

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Single nucleotide polymorphism (SNP) in miRNA binding site of target genes referred to as polymirts can modify, abolish or create a new miRNA binding site and hence is perceived to have significant functional implications in cancers. A typical miRNA-mRNA association involves 6-8 nucleotides base pairing whereas an average human 3/UTR is 950 nucleotides long, allowing one 3/UTR to interact with multiple miRNAs. Subsequently, a SNP in 3/UTR target gene can have much more diverse consequences for their ability to affect multiple miRNAs.[2]Growing data base and high throughput techniques has facilitated identification of such polymirts.[3]

A significant discrepancy is observed in distribution of oral cancers among geographically heterogenous areas, while not a common cancer worldwide it ranks second only to breast cancer among Pakistanis.[4] RAS

(Kirsten rat sarcoma) is frequently mutated oncogene in head and neck cancers among eastern countries.[5] Let-7 miRNAs binds to 3/UTR of KRAS and induces its down regulation. A SNP at Let-7 miRNA binding site of KRAS involving replacement of T with G base creates a variant allele KRAS LCS6 *(rs61764370 T/G).*[6]This variant allele attenuates binding site for Let-7 miRNA at KRAS 3'-UTR with a subsequent KRAS over expression and accelerated carcinogenesis. [6,7,8]

Several studies over recent years have demonstrated impact of this SNP on various attributes of cancer. A reduced survival has been linked to carriers of variant allele in oral cancer[9] and increased risk of lung cancer especially among patients with low dose smoking is observed in variant allele inhabitants.[6]Christensen et al observed increased KRAS expression and decreased Let 7 miRNA expression in their patients of oral cancer. [9]

However, a role of KRAS LCS6 is found in imparting poor prognosis in oral cancer but scarcity of data available from Pakistan stimulated the quest of exploration of outlook of this mutation in predicting prognosis in our patients who inhabit different risk factors. Objective of the study was to explore the frequency of KRAS-LCS6 single nucleotide polymorphism in oral cancer and to explore its diagnostic and prognostic potential.

Materials and Methods

Institutional Review Board of Ziauddin university gave approval for this case control study. Prospective recruitment of cases was done from oncology department of Ziauddin hospital via purposive sampling between 2014 to 2017. Informed written consent was obtained from cases and controls. A history of any other tumor, immune disaese, cerebrovascular or cardiovascular disaese were set as exclusion criteria. Healthyage and sex matched indivisuals not addicted to smoking ciggaretes or chewable products having good oral hygiene were selected as controls.

Sample size was calculated using OpenEpi software. At confidence level of 99 % and power of 90 % the minimum sample size required was 96 for each group. We recruited 200 subjects including 100 cases and 100 controls.

Sociodemographic and clinicopathological data was recorded in a structured questionnaire by taking history, performing physical examination and exploring patient's data files. Staging was done according to AJCC 7th Edition.

DNA was purified from blood samples collected in EDTA vacutainers after careful labeling. DNA was extracted using QIAmp Nucleic acid extraction kit from Qiagen according to manufacturer's protocol. Spectrophotometric measurement of absorbance of extracted DNA at 260-280 was used to determine yield and purity of DNA. The ratio of A260 to A280 of 1.8 to 2.0 was considered as appropriate DNA extraction. A ratio less than 1.8 was suggestive of protein contamination and hence such samples were not included in the study. Successful extraction of DNA was further confirmed by allowing the extracted samples to run through 1 % Gel. After separation, the extracted DNA is seen as visible bands on gel electrophoresis. The extracted DNA were carefully labeled and saved for further analysis.

SNP genotyping was performed via Taqman allelic discrimination assay using set of primers and allele specific probes. Taqman based chemistry is based on 5/ nuclease activity. This detection method is highly specific, sensitive, and reproducible.

Assay is designed with a set of region-specific forward and reverse primer, and allele specific probes capable of detecting wild type and variant allele separately as each probe allows preferential binding of one specific allele, allowing highly specific detection of both alleles with in a single reaction where HEX Probe is specific for wild type allele and FAM probe for variant allele.

Following protocol was developed after optimization, using these sequences of primers and probes ordered from Macrogen.To improve the detection efficiency an initial PCR reaction amplified the target gene which was then amplified using set of allele specific probes.

Forward primer: GCCAGGCTGGTCTCGAA

Reverseprimer:

TGAATAAATGAGTTCTGCAAAACAGGTT

Reporter sequence 1 CTCAAGTGATTCACCCAC-**HEX**

Reporter sequence 2 CAAGTGATGCACCCAC-**FAM**

We added to PCR tubes 10 ul of master mix (Finzyme), 1 ul of forward primer, 1 ul of reverse primer, 5 ul of template DNA, 1 ul each of FAM and HEX probe and 7 ul of water. PCR was run using following settings an initial denaturation 95 C for 3 min, and then 40 cycles 95 C for 40 sec, 57 C for 30 sec, 72 C for 30 sec.

After real time PCR run the PCR curves were visualized for both FAM and HEX to look for the alleles. Allelic discrimination plot was analyzed to look for homozygous versus heterozygous genotypes. Amplified product was run on 2 % Gel to look for any specific bands.

All statistical analysis was performed using the SPSS software package SPSS (Version 24.0; SPSS Inc. Chicago, IL, USA). Chi- square test was used for comparison of nominal and ordinal variables between the groups. Symmetric continuous variables were described as mean with standard deviation and examined using Students t test. P-value of <0.05 was considered as significant.

Results

Mean age at presentation was 45.12 ± 12.42 years for cases (n=100) and 44.22 ±12.2 years for controls (n=100) p>0.05. Oral cancer was more common among males (69 %) compared to females (31 %). Male to female ratio among cases was 2.2:1 and for controls 2.01: 1. Majority of subjects (69%) belonged to Urdu speaking community. Eighty five percent subjects were addicted to smokeless tobacco chewing. Majority (70%) of cases had a moderate differentiation on histopathological examination. Loco regional metastasis to adjacent muscle, bone or skin was observed in 19 % cases. Nodal metastasis was reported in 96 % of cases and distant metastasis seen in 7 %. Seventy two percent patients presented at Stage IV **(Table I).**

Table I: Clinicopathological variables of oral cancer cases

Variables		Casesn=100
Tumor Size	T1	3
	T2	47
	T3	22
	T4	28
Nodal Involvement	Negative	7
	Positive	93
Distant metastasis	Yes	7
	No	93
Loco regional metastasis	Yes	19
	No	81
Tumor Grade	I	14
	II	70
	III	16
Recurrence	Yes	7
	No	93

N; Number of Cases, T1&T2; ≤ 4cm, T3 &T4; > 4cm

Of 200 subjects, including 100 cases and 100 controls none of the controls had variant allele in the KRAS - LCS6 locus. Among cases only 4 had a variant allele G and all 4 were heterozygous. Genotype GG was not detected in any subject giving a minor allele prevalence of 2 %. It is indicated that rs61764370 TG/GG genotype vs TT genotype did not increase risk of developing Oral cancer (P>0.05).

It is not possible to run any statistical test on small number of cases with variant allele but upon studying the cases with variant allele it could be observed that all of them were non-chewers and 3 out of 4 (74 %) had a positive family history. However, no inference can be drawn on such a small frequency. A confirmatory PCR was run on four patients with heterozygous genotype. **Figure 1** displays heterozygous genotype as seen with amplification of both FAM and HEX probes and allelic discrimination plot of the heterozygous subjects.

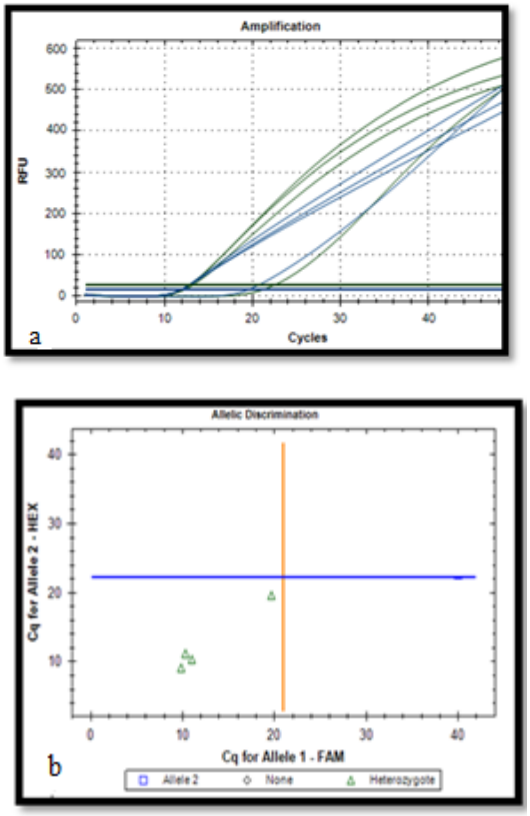

Figure I: KRAS-LCS6 variant allele (Heterozygous genotype) among four patients

Confirmatory Real time PCR carried out on extracted DNA using primers and allele specific probes**.** Presence of green (HEX) curves and blue (FAM) curves confirms heterozygous genotype in four subjects. Straight lines represent non template control showing no expression for either allele. (a)Allelic discrimination plot derived from real time PCR data analysis on a confirmatory PCR for variant allele (b).

Taqman probe-based methodology is a highly specific way of genotyping and does not need gel electrophoresis to confirm specific band sizes for different alleles. However, we ran the amplified product on 2 % gel and observed some faint but interesting bands as seen in **Figure II.**

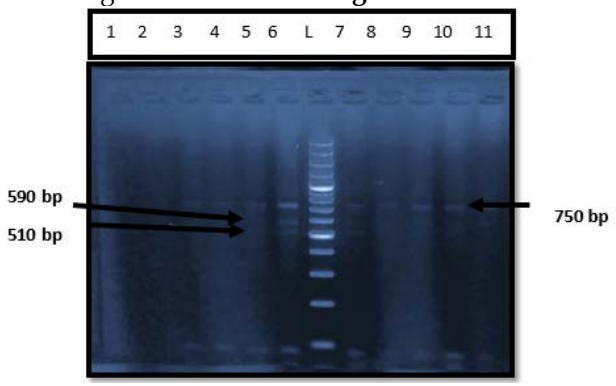

Figure II: Gel Electrophoresis of PCR products of KRAS LCS6 SNP Genotyping (Lane 1, No template control, Lane L, DNA Ladder, Lane 3 to 6 & 7 to 11, Amplified product of KRAS LCS6 showing 750 bp band of wild type T allele, Lane 6 & 7 distinct but faint bands of 590 bp and 510 bp of Variant G allele).

Discussion

KRAS, an oncogene mutated in around 30% of human malignancies contributes to progression of oral cancer which is more substantial in eastern countries compared to west. While it is found to be mutated in only 5 % cases of head and neck cancer its mutation backs to 18 % cases in eastern countries.[5] In a study carried out in Lahore, Pakistan KRAS was significantly over expressed in oral cancer suggesting a role as early diagnostic marker.[10] A single nucleotide polymorphism at Let 7 miRNA binding site in 3/UTR of KRAS has recently been advocated to increase cancer risk and instruct poor prognosis.

To the best of our knowledge this is first report on KRAS LCS 6 polymorphism in oral cancers from Pakistan. Considering the fact that KRAS mutations in oral cancers are more common among Asians we hypothesized a stronger impact of the variant allele on oral cancer risk and survival. To our surprise we observed allele prevalence of only 4 % in our cases and none of the controls had variant allele.

Similar to us Kubova et al in a comprehensive study carried out on breast, colorectal and non-small cell lung cancer did not observe any difference in variant allele prevalence between above cancers and healthy controls in Czech population.[11] Lack of any association was taped up by a metaanalysis based on 30 studies comprising of 14936 cases and 15168 controls confirmed lack of genetic susceptibility to cancer risk and hence potential of being utilized as a biomarker.[12]

Compared to us Christensen et al observed an allele frequency of 19.5 % among cases and 16.3 % among controls in a case control study conducted in Massachussets.[9] Similar to Christensen et al, Wiedhaaset al reported an allele frequency of 16.7 % in a group of head and neck cancer cases in their trial conducted in California.[13] In another study carried out in Brazil an allele prevalence of 17.58 % was reported.[14]

There is no data on variant allele in oral or any other cancer from Pakistan to compare allele frequency. Similar to us irrespective of differences in allele frequencies, none of the above studies concluded a higher risk of head and neck cancer attributed by variant allele. However, the frequency of variant allele is higher in above studies compared to ours which can be explained by the fact that none of these studies was from Asia and a low prevalence of this allele has been testified among Asians.[15] Moreover, it cannot be denied that all above studies were conducted in head and neck cancers which is a broader category including not only oral cancers but also thyroid, nasopharyngeal, salivary gland and laryngeal tumors while our patients were only a subgroup including tumors of oral cavity, lip and tongue. It may also be assumed that variant allele does not increase risk of cancer in normal mucosa, but, in the presence of specific factors if process of carcinogenesis starts it accelerates the process. Such SNPs might be functional in the existence of specific risk factors like alcohol intake, which are clearly different in our patients as only 2 % of our patients were addicted to alcohol.

Christensen et al observed a poor survival in head and neck cancers among variant allele inhabitant which is not witnessed by us again key reason for nonexistence of any observation is the extremely low frequency observed in our patients which limits the ability to draw any conclusion. Another reason could be the fact that our patients were in advanced stage of cancer thus masking the possible effect of variant allele on survival. A better analysis is possible with both early and advanced stage cancer.

We observed that out of 4 patients with variant allele 3 had a positive family history. Such observation has not been reported by any other study and needs to be investigated as again low frequency limits any conclusion from our results.

Chung et al in a study conducted on head and neck cancer patients as well as tumor cell lines from patients with recurrent and metastatic tumors observed allele frequency of 32 %. Apparent reason for the higher prevalence in this cohort is the inclusion of only recurrent and metastatic cases. They further reported a poor response to cisplatin and better response to cetuximab in their patients.[16]

Weidhaas et al in their phase 3 clinical trial on head and neck cancers concluded a good treatment response to cetuximab in variant allele carriers. They further observed a substratum of variant allele who are P16 positive had worst prognosis if not treated with cetuximab. Based on these findings this SNP might be utilized in streamlining personalized treatment protocol with effective tumor targeting and hence improved prognosis.[13]A relatively small sample size and recruitment of patients from single center might be considered as a limitation of this study.

Conclusion

Our analysis suggests that, KRAS-LCS6 (rs61764370) SNP holds no diagnostic or prognostic relevance in oral cancer patients in this cohort.

Recommendations

Identification and exploration of more promising miRNA target gene SNPs by appropriate utilization of bioinformatics is recommended.

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Conflict of interest: None

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Reference

1. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of microRNA biogenesis, mechanisms of actions, and circulation. Frontiers in endocrinology. 2018 Aug 3; 9:402.
2. Saunders MA, Liang H, Li WH. Human polymorphism at microRNAs and microRNA target sites. Proceedings of the National Academy of Sciences. 2007 Feb27;104(9):3300-5.
3. Bhattacharya A, Ziebarth JD, Cui Y. PolymiRTS Database 3.0: linking polymorphisms in microRNAs and their target sites with human diseases and biological pathways. Nucleic acids research. 2014 Jan 1;42(D1):D86-91.
4. https://gco.iarc.fr/today/data/factsheets/populations/586-pakistan-fact-sheets.pdf
5. Kodaz H, Kostek O, Hacioglu MB, Erdogan B, Kodaz CE, Hacibekiroglu I, Turkmen E, Uzunoglu S, Cicin I. Frequency of Ras mutations (Kras, Nras, Hras) in human solid cancer. Breast cancer. 2017;7(12):1-7.
6. Chin LJ, Ratner E, Leng S, Zhai R, Nallur S, Babar I, Muller RU, Straka E, Su L, Burki EA, Crowell RE. A SNP in a let-7 microRNA complementary site in the KRAS 3′ untranslated region increases non–small cell lung cancer risk. Cancer research. 2008 Oct 15;68(20):8535-40.
7. Ratner E, Lu L, Boeke M, Barnett R, Nallur S, Chin LJ, Pelletier C, Blitzblau R, Tassi R, Paranjape T, Hui P. A KRAS-variant in ovarian cancer acts as a genetic marker of cancer risk. Cancer research. 2010 Aug 15; 70(16):6509-15.
8. Paranjape T, Heneghan H, Lindner R, Keane FK, Hoffman A, Hollestelle A, Dorairaj J, Geyda K, Pelletier C, Nallur S, Martens JW. A 3′-untranslated region KRAS variant and triple-negative breast cancer: a case-control and genetic analysis. The lancet oncology. 2011 Apr 1;12(4):377-86.
9. Christensen BC, Moyer BJ, Avissar M, Ouellet LG, Plaza SL, McClean MD, Marsit CJ, Kelsey KT. A let-7 microRNA-binding site polymorphism in the KRAS 3? UTR is associated with reduced survival in OSCC. Carcinogenesis. 2009 Apr 20;30(6):1003-7.
10. Kazmi MM, Qazi MH. Dynamics of Kristen RAS and isoforms of protein kinases in the progression of oral potentially malignant to malignant lesions. Pakistan Oral & Dental Journal. 2014 Mar 1; 34(1).
11. Kubova B, Simova J, Uvirova M, Urbanovska I, Konvalinka D, Mech R, Mazurova J, Hopenstokova A, Dvorackova J. Prevalence of KRAS-LCS6 polymorphism (rs61764370) within three different tumour types (breast, colorectal and non -small cell lung cancer, NSCLC): A study of Czech population. EUROPEAN JOURNAL OF CANCER 2013 Sep 1 (Vol. 49, pp. S202-S202).
12. Zhang SY, Shi J. rs61764370 polymorphism of Kras and risk of cancer in Caucasian population: A meta-analysis. Journal of cancer research and therapeutics. 2016 Apr 1;12(2):699.
13. Weidhaas JB, Harris J, Schaue D, Chen AM, Chin R, Axelrod R, El-Naggar AK, Singh AK, Galloway TJ, Raben D, Wang D. The KRAS-variant and cetuximab response in head and neck squamous cell cancer: a secondary analysis of a randomized clinical trial. JAMA oncology. 2017 Apr 1;3(4):483-91.
14. Bueno Santiago M, de Lima Marson FA, Secolin R, Ribeiro JD, Passos Lima CS, Bertuzzo CS. SLC23A2-05 (rs4987219) and KRAS-LCS6 (rs61764370) polymorphisms in patients with squamous cell carcinoma of the head and neck. Oncology letters. 2014 Jun 1;7(6):1803-11.
15. Sha D, Lee AM, Shi Q, Alberts SR, Sargent DJ et al. Association study of the let-7 miRNA-complementary site variant in the 3′ untranslated region of the KRAS gene in stage III colon cancer (NCCTG N0147 Clinical Trial). Clinl Cancer Res.2014 Jun 15;20(12):3319-27.

16. Chung CH, Lee JW, Slebos RJ, Howard JD, Perez J et al . A 3′-UTR KRAS-variant is associated with cisplatin resistance in patients with recurrent and/or metastatic head and neck squamous cell carcinoma. Ann oncol:2014; 25(11):2230-6.

CONTRIBUTION OF AUTHORS:

- N.M. and S.M. conceived presented idea, developed the theory, performed the computations and wrote manuscript
- Q.J. encouraged to investigate and supervised the findings of this work. N.M, M.H and S.M carried out the experiment. Q.J. and M.H. verified the analytical methods.
- H.A. and R.A. performed the analytic calculations and performed the numerical simulations and tumor grading
- All authors discussed the results and contributed to the final manuscript.