Clinics of Oncology

Case Report ISSN: 2640-1037 | Volume 6

Non-Synonymous Variants of Methylenetetrahydrofolate Reductase C677T Gene Polymorphism showing Discordance with KRAS Mutation in Circulating Tumor Cells of Hepatocellular Carcinoma - A Rare Case Report

Saxena AK*

Department of Pathology/Lab Medicine, Human Cytogenetic and Molecular Genetics Laboratory, All India Institute of Medical Sciences, Patna, Bihar, India

***Corresponding author:**

Ajit Kumar Saxena,
Department of Pathology/Lab Medicine, Human Molecular Genetics Laboratory, All India Institute of Medical Sciences, Patna, 801507, Bihar-India,
E-mail: draksaxena1@rediffmail.com

Received: 15 Jun 2022
Accepted: 25 Jul 2022
Published: 30 Jul 2022
J Short Name: COO

Citation:

Saxena AK. Non-Synonymous Variants of Methylenetetrahydrofolate Reductase C677T Gene Polymorphism showing Discordance with KRAS Mutation in Circulating Tumor Cells of Hepatocellular Carcinoma - A Rare Case Report. Clin Onco. 2022; 6(9): 1-5

Keywords:

Hepatocellular Carcinoma; KRAS protooncogene; MTHFR C677T gene polymorphism

1. Abstract

Circulating Tumor Cells (CTCs) are important aspect for clinical prognosis and diagnosis from primary tumor to metastatic stage during transition of epithelial to mesenchymal stage. The transition of CTCs are characterized by genetic markers such as Sox4, CK-19 and EpCAM using RT-qPCR with the help of specific amplicons. Present cases study of Hepatocellular Carcinoma (HCC) tries to establish the possible link between KRAS oncogene mutation and methylene tetrahydrofolate reductase (MTHFR) C677T gene polymorphism using ARMS-PCR to determine the genetic heterogenicity. Findings reveals that in Tm values shift between case 85.00 and 88.00 GAPDH act as genomic control confirming the substitution of nucleotides from cytosine to thymidine followed by change of amino acids alanine to valine due to point mutation. Simultaneously, the KRAS oncogene showing lack of mutation after using two different sets of amplicons (280bp) to confirm the findings, and suggesting that heterozygous (CT genotype) conditions increase the "risk factor" independently in disease.

2. Introduction

Hepatocellular carcinogenesis (HCC), a multifactorial disease and is regulated by genetics and environmental factors. Several studies have been shown with inconsistent findings regarding variation in the frequency between genotypes (CC, TT) homozygous and CT heterozygous condition of methylene tetrahydrofolate reductase (MTHFR) C677T gene polymorphism [1]. Recent studies have been demonstrated that KRAS mutant - type are (MT) are resistant to wild-type genotypes after chemotherapeutic (cytotoxic) agents in primary or metastatic stages in variety of cancer like colorectal, liver and lungs [2]. The mutational analysis of KRAS is responsible for dysregulation of RAS/MARS mediated signalling pathway and modulate the cell growth, proliferation, and survival. Circulating Tumor Cells (CTCs) play an important role in cancer management to the clinicians for early prognosis and diagnosis based on relevant biomarker during therapeutic regime. Several techniques have been established and showing conflicting findings regarding CTCs number, survival and ~1% sensitivity [3]. The CTCs are originating after detachment from the primary tumour site and circulates in blood stream where they display heterogeneous group of cell population during metastasis. CTCs is a rich source of "liquid biopasy" and act as information centre to monitor cancer. management during metastatic. Epidemiological study reveals that China shows second highest cause of death, but the exact cause of etiopathology is still not clear and certainly epigenetic factors such as hepatitis infection, life-style like use of alcohol with contaminated food also incorporate to increase "risk factor of the disease. The hepatocytes are highly sensitive towards xenobiotics and detoxification process is regulated by couple of enzymes cytochrome 450 and glutathione S transferase [6-8].

Efforts have been developed to improve the CTCs number with maintaining sensitivity using in-vitro techniques after collection

of blood (0.5ml) samples from clinically diagnosed case of HCC. The sensitivity was characterized by Epithelial to Mesenchymal Transition (EMT) markers such as SOX4, EpCAM and CK19 [9]. These markers are highly conserved in nature and extensively used for morphogenetic transformation during metastasis. SOX4 is a member SRY- high mobility homo box region belong to transcription factor, encodes proteins are responsible to decide fate of cell differentiation and their expression is based on activation of oncogene through involving TGF-β signalling [10]. Similarly, epithelial cell adhesion molecule (EpCAM), cytokeratins (CK19 is also known as EMT makers of cell surface of stem cells, whose expression varies during migration at metastatic stage [11, 12]. In the present study, the author tries to explore or to find out the connecting link between KRAS oncogene and MTHFR C677T gene polymorphism in the cases of HCC.

3. Materials and Methods

A 52-year-old male farmer by profession belong to rural population attend the OPD of Department Chemotherapy of All India Institute of Medical Sciences (AIIMS) Patna and referred to Molecular Genetic Laboratory in Department of Pathology/Lab Medicine to assess the genetic profiling. Pedigree analysis shows lack of the family history of cancer. Blood sample (1.5ml) was collected under sterile condition and short-term lymphocytes cultures were set up in triplicates (n=3) as details followed by laboratory procedure earlier in details [13].

CTCs were isolated from cultured lymphocytes samples using Ficoll's gradient methods after mixing (3.0 ml) with Ficoll-Paque plus in glass tube, and centrifuged at 400 x g for 30 minutes at 20°C. After centrifugation, a ring was formed at the junction of plasma and Ficoll's layer. The upper layer was saved gently for isolation of CTCs and genomic DNA was isolated and quantified by nanodrop spectrophotometer. The comparative analysis and sensitivity of CTCs was determined EMT markers (SOX 4, EpCAM &CK19) using RT-PCR [11, 14] and DNA copy number variation (DNA CNV) of individual bands were characterized by densitometry analysis of using software of Gel Doc system (Bio Rad), on 1.5% agarose gel electrophoresis after ethidium bromide staining.

4. Characterization of CTCs markers-SOX 4, EpCAM and CK-19

Table-1, showing the specific EMT forward/reverse primers of SOX4, EpCAM and CK-19 after confirmation of sequences from NCBI (BLAST/http://blast.ncbi.nlm.nih.gov.). PCR reaction was achieved in a 25µl mixture containing 5X Green GoTaq PCR reaction buffer, dNTPs Mix (10 mM), 1µl each of 10 pmol of CTCs specific primer i.e. forward and reverse, 0.2µl of Go Taq DNA polymerase (5U/µl). Genomic template of DNA (50 ng) is mix with reaction mixture before using PCR. The reaction profile was different for each of the CTC's marker i.e. carried out for 35 cycles comprising, initially denaturation at 95°C for 5 minutes. There are three markers showing different PCR protocols on the basis of annealing temperature like SOX4 denaturation at 95°C for 30 seconds, annealing at 57.2°C for 30 seconds, elongation at 72°C for 30 seconds, followed by final elongation at 72°C for 8 minutes, Similarly, for EpCAM, the denaturation at 95°C for 45 seconds, annealing at 58.7°C for 1 minute, elongation at 72°C for 1 minute, followed by final elongation at 72°C for 7 minutes. and CK-19 (Denaturation at 95°C for 45 seconds, annealing at 60.2°C for 30 seconds, elongation at 72°C for 1 minute, followed by final extension at 72°C for 7 minutes) as shown in (Table 1).

Table 1: RT-PCR showing the set of primers (forward/reverse) used for the identification and characterization of EMT markers from Circulating Tumor Cells and specific primers used for MTHFR C677T genes polymorphism in the case of HCC.

S.N.	Oligo Type	Oligonucleotide Sequences (5'-3')	Annealing Temp. (°C)	Amplicon Size (bp)	References
1	SOX4	Forward GGTCTCTAGTTCTTGCACGCTC	57.2	183	[15]
		Reverse CGGAATCGGCACTAAGGAG			
2	EpCAM	Forward GCCAGTGTACTTCAGTTGGTGC	58.7	359	[16]
		Reverse CCCTTCAGGTTTTGCTCTTCTCC			
3	CK 19	Forward ATTCCGCTCCGGGCACCGATCT	60.2	573	[17]
		Reverse CGCTGATCAGCGCCTGGATATGCG			
4	*MTHFR C667T	Forward TGTCATCCCTATTGGCAGGTTACCCCAAA	58	171	[18]
		Reverse CCATGTCGGTGCATGCCTTCACAAAG			
		Cpoly GGCGGGCGGCCGGGAAAAGCTGCGTGATGATGAAATAGG		150	
		T allele GCACTTGAAGGAGAAGGTGTCTGCGGGCGT		105	

*ARMS PCR is used using four different set of primers

4. MTHFR C677T Gene Polymorphism.

MTHFR gene polymorphism analysis was carried out to assess the genetic heterogenicity followed by "risk factors" using ARM-PCR as mentioned earlier [18]. This is highly sensitive, reliable technique used for SNP analysis to detect mutant alleles of MTHFR, based on Tm values to increase the allele specificity of specific primers (tetra plex) as shown in table-1.

To increase the specificity of the reaction, the allele-specific primers were selected and confirmed by software to obtain maximum Tm values.This tetra primer selected for ARMS - PCR of MTH-

FR C677T genotype i.e. CC (wild type) and CT (mutant) either in homozygous and heterozygous condition using SYBR green. The primers used in present study- MTHFR-T, 5' – GCACTTGAAGGAGAAGGTGTCTGCGGGCGT-3'; MT MTHFR-C-poly G, 5'-GGCGGGCGGCCGGGAAAAGCTGCGTGATGATGAAATAGG-3'; MTHFR-cf, 5'-TGTCATCCCTATTGGCAGGTTACCCCAAA-3'; MTHFR-cr, 5' - CCATGTCGGTGCATGCCTTCACAAAG-3'. The reaction mixture consists of a total volume of 20µl containing 10µl of SYBR Green PCR Master mix, 1 µl of each primer per reaction, 40ngm of genomic DNA, and distilled water was used for RT-PCR analysis. PCR protocol initially consist of denaturation step (95 C for 7 min) was followed by amplification and quantification steps repeated for 30 cycles (950C for 10 s, 60 C for 10 s, 72s, with a single fluorescence measurement at the end of the elongation step at 72° curve analysed the data and reaction was terminated by cooling to 40°C. Melting curves (Tm) were constructed by lowering the temperature to 65°C and later increasing the temperature by 0.2 C/s to 98°C to measuring the change of fluorescence consistently. After obtaining Tm values, RT- PCR, a plot was developed between fluorescence versus temperature (dF/dT) for the amplification of candidate gene products and finally measured at 530nm. PCR products were further analysed on agarose gel electrophoresis by evaluating the appearance of additional band consist of 105bp confirming heterozygosity (CT allele) in the case of hepatocarcinoma.

4.1. Results

Present study, of CTCs shows the differential expression of epithelial to mesenchymal transition (EMT) markers - SOX4, EpCAM and CK19 as shown in figure-1. Interestingly, the band of Sox4 (184) shows very light intensity light, when compared with the other markers EpCAM or CK19 as shown in figure-1 lane-1 (arrow) due either conserved nature or activity modulated by MTHFR gene polymorphism because folate is an essential component during malignancy. The study of DNA copy number variations of Sox4 further confirm the findings i.e. the expression and become half as compared to CK19 and EpCAM (figure 1 bars) using densitometry analysis of individual bands of the EMT markers.

Further, the study was extended to assess the genetic heterogeneity of MTHFR C677T gene polymorphism after isolation of gDNA form CTCs. MTHFR gene is a critical enzyme to regulate the folate metabolism followed by DNA methylation in cancer [19]. The Ct values of MTHFR varies in case (24.20) and native control (24.71), and controls (GAPDH) varies from 21.54(case) to 18.77 (control) as shown in figure-2A. Similarly, the Tm values of MTHFR C677T gene varies between cases 85.50 and native control (85.00) and compare the same with GAPDH 88.00 (cases) and controls (88.00) as shown in figure-2B, confirming the genetic heterogeneity due to significantly shift of Tm values from 88.00 to 85.00.

Figure-3, showing lack of KRAS mutation (220bp) between case (lane-1) and control (lane-2) after using two different amplicons of exons 1- set of primers (forward) 5'-AACCTTATGTGTGACATGTTCTA-3' and 5'-TGGTCCTGCACCAGTAAT-3' (reverse). The same findings were again repeat with exon 2 primers forward 5'-ACTGTAATAATCCAGACTGTGTT-3' and reverse 5'-CCCACCTATAATGGTGAATATCT-3 primers (case L3) and control (L4), respectively, to confirm the findings as observed earlier.

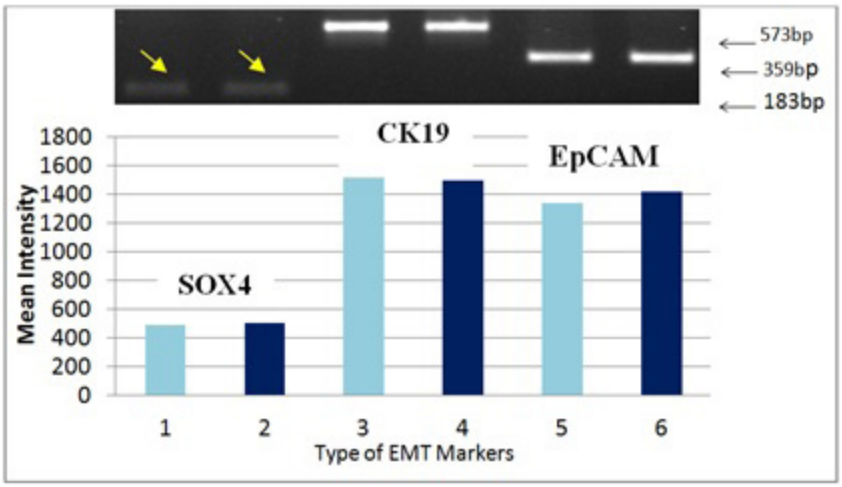

Figure 1: RT-PCR analysis showing the differential expression of EMT markers Sox4 (183bp), Ck19(573) and EpCAM(359bp) between control (L1,3, 5) and case (L2,4,6) after using 1.5 % agarose gel electrophoresis stained after staining with ethidium bromide. Bar diagram showing the DNA copy number variations the case of HCC (light blue = controls: dark blue= case) diagram.

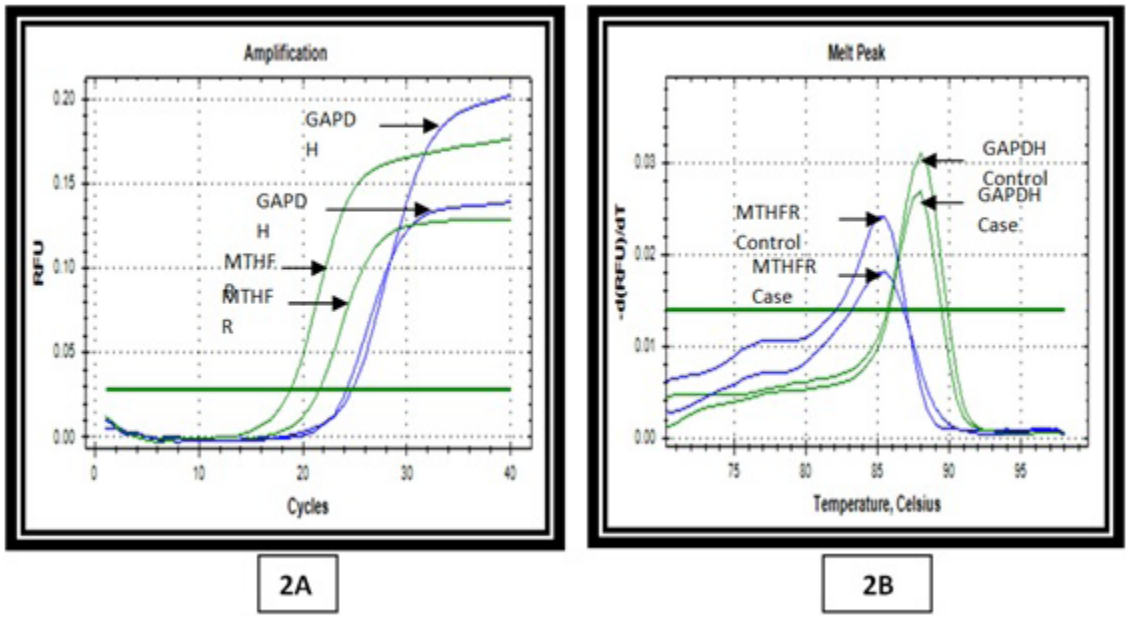

Figure 2A &B: RT-PCR analysis showing MTHFR C677T Gene Polymorphism in HCC, GAPDH (control)

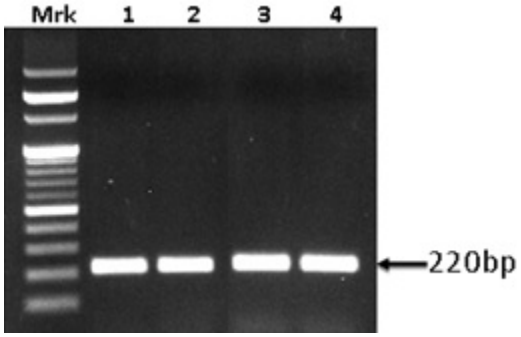

Figure 3: RT - PCR based analysis of KRAS oncogene using two different sets of amplicons (220bp) in the case of hepatocellular carcinoma.

5. Discussion

CTCs are the most important source of "liquid biopsy" for early prognosis and diagnosis. Several techniques have been developed to increase the number and their sensitivity after collection of 5-20 ml of blood samples from the cancer patients [20]. In present study modified techniques is used based on in-vitro culture, where small amount blood samples (0.5 ml) are used in triplicates to isolates CTCs using Ficoll's gradient methods. Further, the sensitivity was characterized using EMT markers (SOX4, EpCAM and CK19) as reported earlier [9]. The significant of this procedure is to collect sample in minimum amount under sterile condition to proliferate cell for 72hrs and compare with non-cultured cells showing the same findings, suggesting to adapt the same procedure in future for the study of CTCs in cancer patient and also save from the ethical issues.

Folate play an important role in cellular proliferation, cell maintenance (repair). During DNA methylation. After, isolation of gDNA was isolated from CTCs to determine heterozygocity of MTHFR C677T gene polymorphism. The large number of studies have been documented in the literature with variable frequency of genotypes (CC, TT) in homozygous condition and heterozygous (CT genotype) condition of MTHFR C677Trs1801133 (C>T) gene. Due to inconsistent findings, author try to synergise the MTHFR C677T gene polymorphism and KRAS oncogene (mutant -type). Interestingly, MTHFR C677T showing the genetic heterogenicity due to significant shifting of Tm values between controls and case of HCC. These changes occurring due to "point" mutation followed by substitution of nucleotide cysteine to thymidine (C→T) resulting changes of amino acid alanine into valine. Earlier study of MTHFR C677T) gene polymorphism shows "new nucleotides variant" based on DNA sequencing including stop codons increase "risk factor"in paediatric tumors [19]. However, the reports on KRAS concordance in metastatic organs other than liver are limited with variable frequency, lungs showing 32.4% when compared to liver (10.2%). However, the few studies have reported up to 50% discordance [20-22], suggesting that these variability in KRAS mutation either due to site specificity or genetic heterogeneity, although, the mechanism behind discordance of KRAS mutation during tumorigenesis is still not clear.

6. Conclusion

Present case study showing lack KRAS mutation either due to tissue or site-specific genetic susceptibility and genetic heterogenicity of MTHFR C677T gene polymorphism increase "risk" of the disease in heterozygous (CT genotype) condition as independent

factor. However, more samples size is required to establish their concordance between two different genes - KRAS oncogene mediated signal transection or MTHFR C677T gene regulating folate metabolism.

References

1. Qi YH, Yao LP, Cui GB et al. Meta-analysis of MTHFR C677T and A1298C gene polymorphism: Association with the risk of hepatocellular carcinoma. Clinics and Research in hepatology and gastroenterology. 2014; 38: 172-80.
2. Kim MJ, Lee HS, Kim YJ, et al. Different metastatic pattern according to the KRA mutational status and site- specific discordance of KRAS status in patient with colorectal cancer. BMC Cancer. 2012; 12: 347-57.
3. Ghossein RA, Bhattacharya S, and Rosai J. Molecular detection of micro metastases and circulating tumor cells in solid tumors. 1999; 5: 1950-60.
4. Donato F, Boffeta P, Puoti M. A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virous infections in causing hepatocellular carcinoma. Int J Cancer. 1998; 75 (3): 347-54.
5. Yu MC, Yuan JM. Environmental factors and the risk for hepatocellular carcinoma. Gastroenterology. 2004; 127(1): 572.
6. Jin F, Qu LS, Shen XZ. Association between methylene tetrahydrofolate reductase C677T polymorphism and hepatocellular carcinoma. Risk: A meta-analysis. Diagn Pathol. 2009; 4(1): 39-46.
7. Imatzuml T, Higaki Y, Hara M, et al. Interaction between Cytochrome 450 1A2 genetic polymorphism and cigarette smoking on the risk of hepatocellular carcinoma in a Japanese population. Carcinogenesis. 2009; 30(10): 1729-34.
8. Chen Yl, Tseng HS,Kuo WH, et al. Glutathione S transferase PI (GSTP1) gene polymorphism increase age related susceptibility to hepatocellular carcinoma . BMC Med Genet. 2010; 11(1): 46.
9. Saxena, Ajit Kumar. Genetic heterogenicity of MTHFR C677T gene polymorphism increase risk factor using circulating tumor cells (CTCs) in acute lymphoblastic anemia - A rare case report. Cancer Medicine Journal. 2022; 5(56): 60-8.
10. Vervoort SJ, Lourenco AR, van Boxtel R, et al. SOX4 mediates TGF β induced expression of mesenchymal markers during mammary cell epithelial to mesenchymal transition. PLos one. 2013; 8 (1): e53238.
11. Trzpis M, McLaughlin PM, de Leij LM, et al. Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. American Journal of Pathology. 2007; 171(2): 386-95.
12. Jain R, Fischer S, Serra S, et al. The use of cytokeratin 19 (CK19) immunohistochemistry in lesions of the pancreas, gastrointestinal tract and liver. Appl Immunohistochem Mol Morphol. 2010; 18(1): 9-15.
13. Saxena A K, Kumar A. Microdeletion of the AZFc locus with high frequency of mosaicism 46, XY/47XYY in cases of non-obstructive azoospermia in eastern population of India. Genetics and Molecular Research. 2019; 18(2): 18349.
14. Mehrpouya M Pourhasshem Z, Yardehnavi N, et al. Evaluation of cytokine 19 as a prognostic tumoral and metastatic markers with focus on improved detection methods. J Cell Physiology. 2019; 1-19.
15. Jafarnejad SM, Wani AA, Martinka M, et al. Prognostic significance of Sox4 expression in human cutaneous melanoma and its role in cell migration and invasion. American Journal of Pathology. 2010; (6): 2741-52.
16. Alowaidi F, Hashimi S M, Alqurashi N, et al. Assessing stemness and proliferation properties of the newly established colon cancer 'stem' cell line, CSC480 and novel approaches to identify dormant cancer cells. Oncology reports. 2018; 39(6): 2881-91.
17. Balducci E, Azzarello G, Valori L, et al. A new nested primer pair improves the specificity of CK-19 mRNA detection by RT-PCR in occult breast cancer cells. The International journal of biological markers. 2005; 20(1): 28-33.
18. Saxena A K, Gupta RK, Kumar M, et al. ARMS-PCR based SNP analysis of MTHFR C677T allele using Syber green in pancreatic tumor. British Journal of Medicine and Medical Research. 2016; 11(12): 1-6.
19. Saxena A K, Tiwari M, Kumar V, Singh K C, et al. MTHFR New Gene Variants Increase Risk Factor in Wilms' tumor and Prediction of 3D Structure Modulates Functional Activity During Drug - Protein Interaction. Journal of Integrative Oncology. 2020; 11(12): 1-6.
20. Politaki E, Agelali S, Apostolaki S, et al. A comparison of three methods for the detection of circulating tumor cells in the patients with early and metastatic Breast Cancer. Cell Physiology Biochemistry. 2017; 44: 594-606.
21. Knijn N, Mekenkamp IJ, Klomp M et al. KRAS mutation analysis: A comparison between primary tumours and matched liver metastasis in 305 colorectal cancer patients. British J Cancer. 2011; 104 (6): 1020-6.
22. Nash GM, Gimbel M, Shia J, et al. KRAS mutation correlates with accelerated metastatic progression in patients with colorectal liver metastasises Ann Surg Oncol. 2010; 17(2): 572-8.