Association of rs1042522 SNP with Clinicopathologic Factors of Breast Cancer Patients in the Markazi Province of Iran

Ali Arash Anoushirvani1, Reza Aghabozorgi1, Azam Ahmadi2*, Mohammad Arjomandzadegan2, Maryam Sahraei2, Sara Khalili2, Taha Fereydouni2, Zoha Khademi2

1Khansari Hospital and Department of Internal Medicine, School of Medicine, Arak University of Medical Sciences, Arak, Iran; 2Infectious Diseases Research Center (IDRC), Arak University of Medical Sciences, Arak, Iran

Abstract

BACKGROUND: The nucleotide changes in different genetic loci increased the incidence risk of breast cancer.

AIM: The aim of present study was to investigate genotype distribution at codon 72 of the TP53 gene (rs1042522) in breast cancer patients to achieve a potential diagnostic marker related to some demographic feathures.

METHODS: In our case-control study, blood samples were collected from a total of 34 patients harboured breast cancer. DNA was extracted, and nested-PCR was performed. Products were digested with AccII and subsequently were sequenced. Results were compared with samples characteristics.

RESULTS: The PCR results indicated the correct implementation of extraction and amplification protocol. The genotypic distribution at codon 72 of TP53 in control group was 20%, 62.4% and 16.6% for Arg (homozygous), 50% heterozygous, and 26.47% (heterozygous) and Pro (homozygous variant) respectively. Also, this distribution in the patient group was 23.52% (homozygous variant), 50% heterozygous, and 26.47% another homozygous variant (Adjusted odds ratio: 1.12 and 95%CI = 0.57 to 2.2, P = 0.03). The absence of Arg at codon 72 of TP53 is relevant with age higher than 40 years and metastasis to other organs.

CONCLUSION: Polymorphism at codon 72 of TP53 was associated with high-grades of breast cancer risk and different responses to chemotherapy treatment. It is recommended genotype distribution of codon 72 of TP53 before chemotherapy.

Introduction

Breast cancer (BC) is one of the most common types of cancer that causes mortality rates each year. Despite advances in early diagnosis and proper treatment for this disease, it remains the main cause of death among women [1], [2], [3]. The most common sites of BC metastasis are bones, lunge and liver. Metastasis of BC to the brain less occur. The growth of cancer cells in BC is considered in three stages. BCs stage I or II are in the initial stage. If the tumour extends to the underlying muscles of the chest wall or skin, BC is type III. This Stage also includes BCs involving inflammatory system, in which case the breast is red or swollen [4], [5], [6]. BC stage IV refers to tumours that metastasise to the outside of the breast and the lymph nodes, as well as to the brain, bones, skin, and other organs. The basics of cancer genetics is the occurrence of changes in signalling pathways and genes regulatory networks [5]. Changes in different codons of the genes, including
In his...TP53 and tumour grade, age, Body mass index (BMI) and the response rate to chemotherapy in BC patients of Markazi province of Iranian population. The aim of present study was to evaluate the polymorphism at codon 72 of TP53 in patients with BC and investigate the relationship between genotype distributions with above factors in patients with BC in aforementioned Iranian population.

Materials and Methods

In present case-control study, samples were collected from Ayatollah-Khansari hospital in Arak city. This study was performed between November 2016 and June 2017. A total of 34 pathological and clinical data were classified for the collected breast cancer samples (median age: 48 years). Also, normal samples from non-tumour individuals were equal numbers of patients group. Three ml of blood was collected from each sample in CBC test vial. The study was approved by the Research Ethics Committee of Arak University of Medical Sciences (Ethics number 618B/3090). Participants of present study agreed with and signed a consent form.

DNA extraction was performed from 500 μl whole blood using the Diatom DNA Kit (IsoGene, Moscow, Russia) based on its instruction. Samples were loaded on 1% agarose gel, (YTA, Iran). In Polymerase chain reaction, 50 mM MgCl2 (Cinagene, Iran), 10X Buffer (Cinagene, Iran), 10 mM dNTP (Cinagene, Iran), 1unit Taq polymerase (Cinagene, Iran), and specific primers at a suitable concentration of 10 pmol were used. Annealing temperature for the primers [9] was optimised at 52.7°C.

In this study, PCR products of the first stage were used in a thermocycler machine (Eppendorf, Germany) as a template in the second stage PCR using a pair of internal primers in order to more specificity and enhancing the amplified bands. A non-template sample was used as a negative control. The sequence of these primers is shown in Table 1.

| Primer ID | Sequence (5'-3') |
|-----------|-----------------|
| p53 F     | GCTCTTTCACCCCATCTACAG |
| p53 R     | TGAAGCTCATGGAAAGCCAGC |
| p53 Inner-F | TCCCCCTTGCGCTCCTAAA  |
| p53 Inner-R | CGTCAAGTCAGAGACTT   |

Restriction enzyme digestion was performed by Accl (Vivantis, Malasia) at 37°C for 3-16 hours. Production of one fragment of 300bp indicated the presence of the wildtype allele (Arg). The presence of the variant allele (Pro) was determined by the observation of two fragments of 160 bp and 140bp. The produced amplicons were sent for sequencing.
The sequencing was carried out with the ABI Applied Biosystem machine-Model 3730XL (Macrogen, South Korea). Sequencing results were analysed using Chromas, Mega 4.0, Blast and Blat software.

Validation of genotypes was evaluated by comparison of sequences results with data banks such as UCSC. Chi-sq Hardy-Weinberg equilibrium (HWE) test calculator were used for biallelic markers including SNPs. The difference between the groups was determined by one-way ANOVA GraphPad prism 7.0. P values less than 0.05 were considered statistically significant.

Results

The results of the amplification reaction indicated the accurate of the extracted DNA, the correct implementation of the temperature protocol and used primers. Figure 2 shows the 300bp amplicon of the TP53 gene.

![Figure 2: Steps of Nested-PCR steps in this study. The yellow arrow shows the 300 bp amplicon of TP53 gene](image)

In below figure columns 1-4 indicated bands of heterozygous samples that have three bands (300, 160 and 140 bp). Also, Figure 3b shows the sequence analysis of the 8 homozygous amplicons.

![Figure 3: A) Agarose gel of digestion reaction. Column 1-4 showed heterozygote samples (Arg/Pro) and column 5 showed a homozygote wildtype (Arg); B) Nucleotide sequences from the sequencing of clinical samples (Chromas and Mega4 software)](image)

The genotypic distribution in the patient group was 23.52% homozygous wildtype (Arg), 50% heterozygous (Arg/Pro), and 26.47% another homozygous variant (Pro). These genotypes in the control group were 20%, 62.4% and 16.6% for homozygous wildtype, heterozygous and homozygous variant respectively.

The C allele freq = 0.48; G allele freq = 0.52 was the frequency of changes in codon 72 of the TP53 gene in patient group (HWE calculator, p < 0.05). The Chi-square in control group was not significant (X2 = 6.8, p > 0.05).

| Pattern of Band (bp) | Genotype | Patients (%) | Odds Ratio | 90 CI | 95 CI | 99 CI | P value |
|----------------------|----------|--------------|------------|-------|-------|-------|---------|
| Pattern A 300        | CC homozygote | 63.52 | 1.12 | 0.64-1.98 | 0.57-2.22 | 0.48-2.49 | < 0.05 |
| Pattern B 300, 160   | CC homozygote | 50   | 1.07 | 0.66-1.71 | 0.61-1.87 | 0.54-2.23 | < 0.05 |
| Pattern C 160, 140   | CC homozygote | 26.47 | 1.41 | 0.79-2.51 | 0.71-2.78 | 0.57-3.48 | < 0.05 |

The Figure 4 showed that there are a significant association between the changing at codon 72 (Arg72 Pro) of TP53 gene sequences and the Age > 40, BMI > 22, WHR > 0.8, metastasis to other organs, positive ER and PR status and less response to chemotherapy in BC samples than the control group. These associations are statistically significant.

![Figure 4: A) This chart shows the relationship between ampicons sequences and the characteristics of the used clinical samples; B) GraphPad Prism 7.0 software showed association between the Pro at codon 72 of TP53 gene and the Age > 40, BMI > 22, WHR > 0.8 and negative response to chemotherapy (One-way ANOVA test, P = 0.03)](image)

Discussion

The incidence rates of BC increase with an ascending trend so that its frequency has doubled over the past 20 years. In Iran, BC is the most common type of cancer after stomach and oesophagus cancers. Therefore, diagnosis at first stages and study of the molecular mechanisms of BC are necessary [1], [2], [3]. Molecular events that regulate cell survival, apoptosis, growth and differentiation of the cell, played an important role in the overall kinetics of tumour growth as benign or
malignant [14]. Mutation of TP53 is the most common genetic change in human cancers and is associated with poor response to treatment including chemotherapy and radiotherapy. Recent studies have shown the probably beneficial and valuable effects of gene therapy with the goal of TP53 as a complementary therapy in cancers. Studies in 1997 and 1998 found that p53 plays a critical role in controlling of the cell cycle since its normal function not forces the cell to stop the cell cycle so that it can enter a period of interruption to repair the DNA damage. If the cell could not repair damage to the cell, it would commit suicide to prevent mutations in the cells [6], [7]. Injury to the TP53 gene occurs during the life-span of the individual, but in rare cases, involved about 1% the cases of sporadic BCs [15], [16], [17], [18]. In a study in 2009, numbers of 1836 articles from 1986 to 2008 were reviewed for involved genes in BC [3]. In the TP53 gene, the importance of codon 72 has been shown to identify changes in the various populations. This codon site is within the active site of the p53 protein. In studies conducted in 2006 and 2007, it was found that using some of the pathological characteristics of BC, such as the tumor size, negative status of PR, positive status of the lymph nodes and high differentiation can be predicted that a patient will be more likely to develop metastasis [19], [20], [21], [22]. Other studies showed significant differences in the prevalence of codon 72 in TP53 polymorphism in endometriosis in a Brazilian population [23].

The present study showed that the frequency of changes in codon 72 of the TP53 gene in the studied population was common and totally 76.7 % of the studied patients had this change. Also, there are associations between a polymorphism at microRNA binding site in BRCA2 with BC susceptibility [24], [25]. Also, there are an association of polymorphisms in FGFR2 and FGFR3 with a degree of Trastuzumab in the Adjuvant treatment of ERBB2/HER2- positive BC [26]. A study results at 2017 showed a significantly association between six SNPs including FGFR2 (rs2981582), HCN1 (rs981782), MAP3K1 (rs889312), TOX3 (rs3803662), ZNF365 (rs10822013), and RAD51B (rs3784099), with breast cancer risk [25]. A study in South Korea on persons with gastric cancer that treated with cisplatin and paclitaxel chemotherapy indicated that rs1042522 (G/G and C/G) genotypes compared to rs1042522 (C/C) were significantly associated with lower response rate to the chemotherapy treatment (35.7 vs. 66.7%, p-value 0.019) [28]. Another study at 2014 on patients with NSCLC showed that G/G genotype of rs1042522 was more resistant to the first-line chemotherapy drugs, such as cisplatin [29]. In the present study, we evaluated all changes in studied amplicon compared to the control samples by sequencing method. The results of our study indicated the relationship between the rs1042522 polymorphism with age, BMI, metastasis, positive status of PR and ER and treatment response rate in these patients (Table 3).

In the present study, we investigated that rs1042522 SNP may be associated with susceptibility to BC among Iranian women with higher age and BMI. The distribution of C Allele in individuals with age of higher than 40 years was 1.2 times more than lower ages. Also, the frequency of G allele is associated to lower BMI and WHR. Our data showed there was a direct association between C/G and C/G genotype of rs1042522 with a negative response to chemotherapy (1.13 times).

Based on the new issue of “personalised medicine” in modern medicine, patients can be treated according to their molecular feathers. Therefore, it can be inferred that determination of TP53 variants for BC patients is suitable before the starting the treatment protocol. Cancer is a multi-factorial disease and, in addition to genetic, environmental factors such as smoking, family income etc. also contributes to its development. The results of this study revealed the lack of significant correlation between these factors including family income, Educational level, the age of marriage and cigarette smoking with the studied polymorphism (data not shown). However, any of these factors may be effective in the overall survival rate and severity of the disease.

However any of these factors may be effective in overall survival rate and severity of the disease. Performance of molecular diagnostic tests for the evaluation of cancer genes in medical centers is critical. Hence, the determination of polymorphism in the mentioned codon can be used as a potential candidate diagnostic marker for high risk BC.

### Table 3: Some demographic features of the studied samples and differences in genotypes distribution of TP53 at codon 72 in this study (P value < 0.05)

| Characteristic | Detail | Total no. | No. of patients (%) | Sg, Cg, Cc (%) | Di. | Ge, Cg, Cc (%) | Dr. | Gc, Cg, Cc (%) | Dr. | Cc, Cc (%) | Dr. |
|---------------|--------|-----------|---------------------|---------------|-----|---------------|-----|---------------|-----|-----------|-----|
| Age <40       |        | 8         | 23.52               | 8.92          | 16.8| 11.76         | 38.24| 2.94          | 52.06|
| Age >40       |        | 26        | 76.48               | 14.07         | -10.3| 38.23         | -11.17| 23.52         | -1.48|
| Menopausal    |        | 16        | 47.05               | 11.76         | -10.3| 23.52         | -26.48| 23.52         | -1.48|
| Menopausal age | <52    | 3         | 8.22                | 2.94          | -13.24| 2.94          | -47.06| 2.94          | -22.06|
| Menopausal age | >52    | 15        | 44.11               | 8.92          | -22.06| 17.64         | -32.36| 17.64         | -7.36|
| ER status     | Positive | 8         | 25.32               | 5.98          | -19.12| 11.76         | -38.24| 5.88          | -19.12|
| ER status     | Negative | 26        | 76.48               | 17.64         | -7.36| 25.29         | -14.71| 23.52         | -1.48|
| Pr status     | Positive | 8         | 25.32               | 5.98          | -19.12| 11.76         | -38.24| 5.88          | -19.12|
| Pr status     | Negative | 26        | 76.48               | 17.64         | -7.36| 25.29         | -14.71| 23.52         | -1.48|
| Her2 status   | Positive | 15        | 44.11               | 11.76         | -13.24| 20.58         | -29.42| 11.76         | -13.24|
| Her2 status   | Negative | 19        | 55.86               | 11.76         | -13.24| 26.47         | 23.43| 17.64         | -7.36|
| Metastasis    | Yes      | 6         | 17.64               | 5.98          | -19.12| 8.62          | -41.18| 2.94          | -22.06|
| Metastasis    | No       | 28        | 82.36               | 17.64         | -7.36| 41.17         | -8.63| 23.52         | -1.48|
| Treatment     | Chemo-therapy | 6 | 17.64 | 2.94 | -22.06 | 8.62 | -41.18 | 5.88 | -19.12 |

**Di.** the difference between expected and observed genotypes in HWE equation, **p2**: GG, 2pq: Gc, 2qq: Cc

### Acknowledgements

The authors of this article thank for the research and technology deputy of Arak University of medical sciences.

2280 https://www.id-press.eu/mjms/index
Author contributions

The first two authors contributed to this study and have equal role: clinical sample preparation; AA: designed study, performed the experiments, analysed data, interpreted data and manuscript preparation; TA: clinical sample preparation, SA, MA and MS: performed the experiments

References

1. Yarnold J. Early and locally advanced breast cancer: diagnosis and treatment National Institute for Health and Clinical Excellence guideline 2009. Clinical Oncology. 2009; 21(3):159-60. https://doi.org/10.1016/j.clon.2008.12.008 PMid:19167201
2. Israelyan AH. The development of molecular diagnostics for breast cancer. 2003.
3. McCafferty MP, Healy NA, Kerin MJ. Breast cancer subtypes and molecular biomarkers. Diagnostic Histopathology. 2009; 15(10):485-9. https://doi.org/10.1016/j.dhri.2009.07.002
4. Blankrion C, Goldstein LD, Thorne NP, Sipateri I, Chin NF, Dunning MJ, Barbosa-Morais NL, Teschendorff AE, Green AR, Ellis IO, Tavaré S. MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biology. 2007; 8(10):R214. https://doi.org/10.1186/gb-2007-8-10-r214 PMid:17922931 PMCid:PMC2246288
5. Ahmadi A, Khansarinejad B, Hosseinkhani S, Ghaneli M, Mowlia SJ. miR-199a-5p and miR-495 target GRP78 within UPR pathway of lung cancer. Gene. 2017 Jul 15;680:15-22. https://doi.org/10.1016/j.gene.2017.03.032 PMid:28363780
6. Joensuu K. Tumor Dormancy in Breast Cancer. 2012.
7. Mehta D, Gonik M, Klengel T, Rex-Haffter M, Menke A, Rubel J, Mercer KB, Pütz B, Bradley B, Holsober F, Ressler KJ. Using polymorphisms in FKBP polymorphisms in FKBP and MDM2 SNP309 with clinical outcome. International Journal of the American Cancer Society. 2008; 113(4):799-807. https://doi.org/10.1016/j.breast.2007.09.014 PMid:18256690
8. Savad S, Mehdipour P, Miryounesi M, Shirkoohi R, Feredeoooni F, Mansouri F, Modarressi MH. Expression analysis of MiR-21, MiR-205, and MiR-342 in breast cancer in Iran. Asian Pacific Journal of Cancer Prevention. 2012; 13:873-877. https://doi.org/10.7314/APJCP.2012.13.3.873 PMid:22631664
9. Doosti A, Dehkhordi PG, Davoudi N. A p53 codon 72 polymorphism associated with breast cancer in Iranian patients. African Journal of Pharmacy and Pharmacology. 2011; 5(10):1278-1281. https://doi.org/10.5897/AJPP.10.394
10. Han JY, Lee GK, Jang DH, Lee SY, Lee JS. Association of p53 codon 72 polymorphism and MDM2 SNP309 with clinical outcome of advanced nonsmall cell lung cancer. Cancer: Interdisciplinary journal of research. 2007; 121(3):799-807. https://doi.org/10.1002/cncr.20815 PMid:18618574
11. Reiling E, Lyssenko V, Boer JM, Imholz S, Verschuren WMM, Iosoma B, Tuomi T, Groop L, Dörle ME. Codon 72 polymorphism (rs1042522) of TP53 is associated with changes in diastolic blood pressure and risk of coronary heart disease. European Journal of Human Genetics. 2012; 20(6):696. https://doi.org/10.1038/ejhg.2011.240 PMid:22189267 PMCid:PMC3555249
12. Asadi M, Shanjehbandi D, Zarintan A, Pedram N, Baradaran B, Zafari V, Shirmohamadi M, Hashemzadeh S. TP53 Gene Pro72Arg (rs1042522) Single Nucleotide Polymorphism as Not a Risk Factor for Colorectal Cancer in the Iranian Azari Population. Asian Pacific journal of cancer prevention: APJCP. 2017; 18(12):3423. PMid:29286614
13. He J, Wang F, Zhu J, Zhang Z, Zou Y, Zhang R, Yang T, Xia H. The TP53 gene rs1042522 C> G polymorphism and neuroblastoma risk in Chinese children. Aging (Albany NY). 2017; 9(3):852. https://doi.org/10.18632/aging.101196 PMid:28275206 PMCid:PMC5391235
14. Jahani M, Anoushiravani AA, Shahi F, Azimaraqhi O. Abducens nerve palsy as initial presentation of Burkitt Lymphoma during Pregnancy Post–Cesarean abducens nerve paresis and Headache. International Journal of Hematology-Oncology and Stem Cell Research. 2009; 9(1):37-9.
15. De Jong MM, Nolte IM, Te Meeram GJ, Van der Graaf WTA, Oosterwijk JC, Kleibeuker JH, Schaapveld M, De Vries EGE. Genes other than BRCA1 and BRCA2 involved in breast cancer susceptibility. Journal of medical genetics. 2002; 39(4):225-242. https://doi.org/10.1136/jmg.39.4.225 PMid:11950848 PMCid:PMC1735082
16. Osborne C, Wilson P, Tripathy D. Oncogenes and tumor suppressor genes in breast cancer: potential diagnostic and therapeutic applications. The oncologist. 2004; 9(4):361-377. https://doi.org/10.1016/s1354-9378(04)00113-0 PMid:15051505
17. Shojaei S, Gardaneh M, Rahimi Shamabadi A. Target points in trastuzumab resistance. International journal of breast cancer. 2012; 2012.
18. Cristofanilli M, Hortobagyi GN. Molecular targets in breast cancer: current status and future directions. Endocrine-related cancer. 2002; 9(4):249-266. https://doi.org/10.1077/errc.2002.0090249 PMid:12542402
19. Evans DG, Brentnall A, Byers H, Harkness E, Stavrinos P, Howell A, Newman WG, Cuzick J. FH-risk study Group. The impact of a panel of 18 SNPs on breast cancer risk in women attending a UK familial screening study: a case–control study. Journal of medical genetics. 2015; 52(4):111-113. https://doi.org/10.1136/jmedgenet-2015-104125 PMid:27794048
20. Furrer D, Lemieux J, Côté MA, Provencher L, Lafortune C, Barabé F, Jacobs S, Michaud A, Diorio C. Evaluation of human epidermal growth factor receptor 2 (HER2) single nucleotide polymorphisms (SNPs) in normal and breast tumor tissues and their link with breast cancer prognostic factors. The Breast. 2016; 30:191-196. https://doi.org/10.1016/j.breast.2016.09.014 PMid:27788409
21. Hamdi Y, Soucy P, Adoue V, Michailidou K, Canisius S, Lemaçon A, Droit A, Andrulis IL, Anton-Culver H, Arndt V, Baynes C. Association of breast cancer related gene with genetic variants showing differential allelic expression: Identification of a novel breast cancer susceptibility locus at 4q21. Oncotarget. 2016; 7(49):80140. https://doi.org/10.18632/oncotarget.12818 PMid:27792995 PMCid:PMC5340257
22. Toomey S, Madden SF, Furney SJ, Fan Y, McCormack M, Stapleton C, Cremona M, Caballeri GL, Milweska M, Elster N. Carr A. The impact of ERBB-family germline single nucleotide polymorphisms on survival response to adjuvant trastuzumab treatment in HER2-positive breast cancer. Oncotarget. 2016; 7(46):75518. https://doi.org/10.18632/oncotarget.12782 PMid:27776352 PMCid:PMC5342757
23. Camargo-Kosugi CMO, D’Amora P, Kleine JP, Carvalho CVD, Sato H, Schor E, Silva ID. TP53 gene polymorphisms at codons 11, 72, and 248 and association with endometriosis in a Brazilian population. Genet Mol Res. 2014; 13(3):6503. https://doi.org/10.4238/2014.August.26.1 PMid:25177931
24. Cao J, Luo C, Yan R, Peng R, Wang K, Wang P, Ye H, Song C. rs15869 at miRNA binding site in BRCA2 is associated with breast cancer susceptibility. Journal of cancer. 2014; 5(18):377-383. https://doi.org/10.7153/jca-04-016 PMid:26052376 PMCid:PMC4023739
25. Gawin PG, Song N, Kim SR, Lipchik C, Johnson NL, Bandos H, Finnigan M, Rastogi P, Fehrenbacher L, Mamounas EP, Swain SM. Association of polymorphisms in FGFR2A and FGFR3A with degree of trastuzumab benefit in the adjuvant treatment of ERBB2/HER2-positive breast cancer: analysis of the NSABP B-31
26. Anoushirvani AA, Ahmadi A, Aghabozorgi R, Khalili S, Sahraei M, Fereydouni T, Khademi Z. Gengenotypic Evaluation of Hsa-miR-433-3p Binding Site in the Regulatory Region of TYMS in Breast Cancer Patients. AMUJ. 2018; 19.

27. Yi-Chen H, Shih-Hsin T, Chien-Tien S, Er-Chieh C, Chih-Hsiung W, Mao-Chih H, Shiying-Yu L, Yun-Ru L, Chin-Sheng H, Hung-Yi C. A polygenic risk scores for breast cancer risk in a Taiwanese population. Breast cancer research and treatment. 2017; 163(1):131-138. https://doi.org/10.1007/s10549-017-4144-5 PMid:28205043

28. Kim JG, Sohn SK, Chae YS, Song HS, Kwon KY, Do YR, Kim MK, Lee KH, Hyun MS, Lee WS, Sohn CH. TP53 codon 72 polymorphism associated with prognosis in patients with advanced gastric cancer treated with paclitaxel and cisplatin. Cancer chemotherapy and pharmacology. 2009; 64(2):355-60. https://doi.org/10.1007/s00280-008-0879-3 PMid:19052714

29. Zheng D, Chen Y, Gao C, Wei Y, Cao G, Lu N, Hou Y, Jiang X, Wang J. Polymorphisms of p53 and MDM2 genes are associated with severe toxicities in patients with non-small cell lung cancer. Cancer biology & therapy. 2014; 15(11):1542-51. https://doi.org/10.4161/15384047.2014.956599 PMid:25482940 PMcid:PMC4623062