Annals of Diagnostic Pathology 46 (2020) 151482

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

journal homepage: www.elsevier.com/locate/anndiagpath

Original Contribution

MAPK7 variants related to prognosis and chemotherapy response in osteosarcoma

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ARTICLE INFO

Keywords:
Osteosarcoma
Gene sequencing
MAPK7 gene
Prognostic marker
SNPs

ABSTRACT

Osteosarcoma (OS) is a class of cancer originating from the bone, affecting mainly children and young adults. Our previous study showed that MAPK7 gene overexpression was significantly associated with tumor progression, poor treatment response, and worse overall survival, suggesting that MAPK7 could play an important role in OS tumorigenesis. We have investigated if MAPK7 overexpression was a result of any genomic changes in OS tumor specimens. We identified five SNPs (Single Nucleotide Polymorphism) previously described in databases, dbSNP and COSMIC, and identified two single nucleotide substitution not yet described. We found, in pre-chemotherapy specimens, a significant association of MAPK7 rs2233072G allele variant with metastasis at diagnosis and relapse (0.0909 and 0.0455, respectively). In post-chemotherapy, rs1054206GG specimen's genotype was associated with osteoblastic histological type (P= 0.0249) and presented decreased MAPK7 gene expression when compared with pre-chemotherapy specimens of same patients (P = 0.0095). Interestingly, it was observed some SNPs genotype exchange after chemotherapy. Our data indicated that MAPK7 gene expression associated with genotype exchange after chemotherapy, and these SNPs associated with important clinical parameters might be a valuable indicator for predicting in OS.

1. Introduction

Osteosarcoma (OS) is a malignant bone tumor derived from primitive mesenchyme and presents the highest incidence in adolescents between 15 and 19 years. OS is characterized by a high risk of metastasis and the lung is the most frequent site [1]. Approximately 15–20% of OS patients present metastatic disease at diagnosis [2,3] and, despite the successful control of the primary tumor, death by lung metastasis occurs in over 70% of patients [4]. Drug resistance and unfavorable clinical outcome remain problems that affect approximately 50% of patients [5]. In our previous study, it was observe association between *MAPK7* gene overexpression and tumor progression, poor treatment response and worse overall survival [6]. Besides, a functional study of our group demonstrated that silencing *MAPK7* reduced proliferation, migration and invasion in OS cell lines, furthermore, the chemotherapy exposition decreased *MAPK7* expression [7]. Our recent findings suggested that *MAPK7* could play an important role in OS tumorigenesis and treatment response, making this gene an investigative target [6,7]. Our present study investigated, for the first time in the literature, if structural genomic alterations in *MAPK7* gene, such as SNP (Single Nucleotide Polymorphism), mutation, insertion or deletion in pre and post chemotherapy specimens could be related to its expression and thus related to tumorigenesis and progression in OS.

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https://doi.org/10.1016/j.anndiagpath.2020.151482

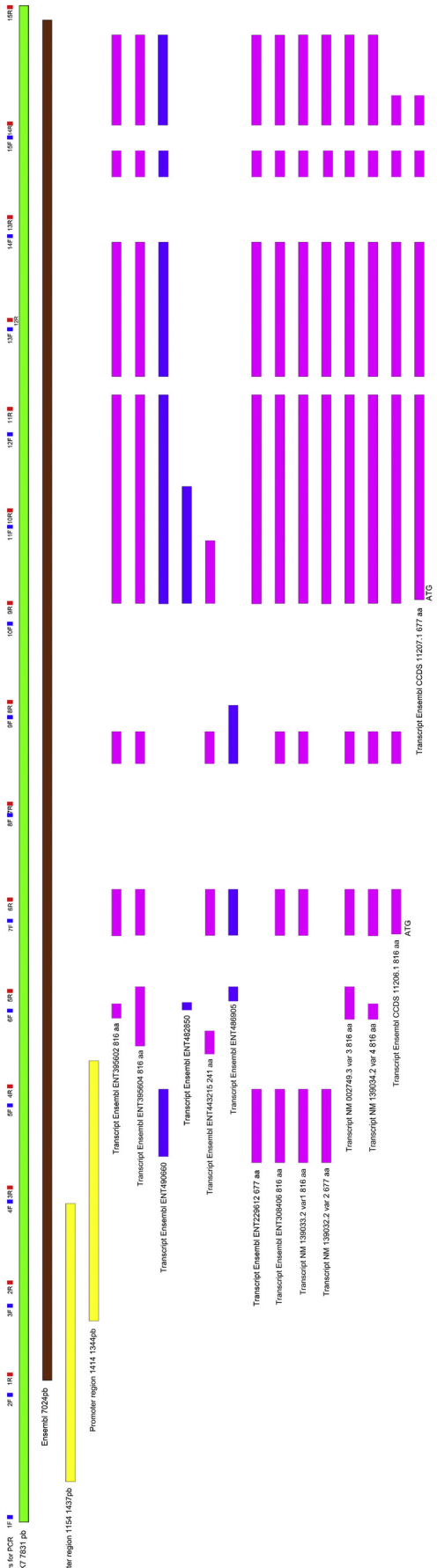

Fig. 1. Design overview and localization of the PCR primers for *MAPK7* gene sequencing. Drawing shows all gene transcripts and the MAPK7 7831 bp sequence.

2. Material and methods

2.1. OS patients

Thirty OS frozen tissues, 15 pre-chemotherapy (Pre-Ch) and 15 post-chemotherapy (Post-Ch) specimens, were obtained from 15 patients that presented *MAPK7* gene overexpression after chemotherapy in our previous investigation [6]. Besides four normal bone specimens were use as calibrator. The normal bone specimens were obtained from healthy individuals without genetic and/or musculoskeletal diseases who underwent orthopedic surgery due to trauma. All specimens were obtained from Biobank of Pediatric Oncology Institute – IOP/GRAACC/UNIFESP (CONEP B-053), and informed consent has been obtained from all individuals.

2.2. OS cell lines

Three OS cell lines (Saos-2, KHOS and M-OS) were investigated. Saos2 and KHOS were purchased from the American Type Culture Collection (Rockville, MD, USA). M-OS is a cell line established in our laboratory from pulmonary metastasis of metastatic OS patient at diagnosis [8]. The cells was cultivated in DEMEM medium containing 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, BRA) in an humidified incubator at 37 °C in 5% CO_2.

2.3. MAPK7 gene expression

Total RNA of 34 fragments (15 Pre-Ch, 15 Post-Ch and 4 normal bone) was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the RNA of cell lines was extracted using NucleoSpin® TriPrep kit (Macherey-Nagel, Duren Germany). The RNA molecules were quantified in NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific) and considered good quality when A260/280 = 2.0. The ideal RNA concentration for each cDNA synthesis was 1 μg/μl. Reverse transcription was performed using oligo primer and Superscript III (Invitrogen, Carlsbad, CA, USA). The *MAPK7* mRNA levels were determined by quantitative real-time PCR (qRT-PCR) using TaqMan gene expression assay (Hs00177079_m1) (Applied Biosystems, Inc., Foster City, CA, USA). *GAPDH* gene (Hs03929097_g1) was used as endogenous control. This analysis was performed in the thermocycler Applied Biosystems Prism 7500 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA, USA). Reactions were done in triplicate. Relative Quantification was calculated by $2^{-\Delta\Delta Ct}$ method [9].

2.4. MAPK7 gene sequencing

The DNA tumor fragment from 34 specimens was extracted using the NucleoSpin DNATrace kit (Macherey-Nagel, Duren, Germany) combined with the NucleoSpin DNA Trace Bone Buffer Set kit (specifical kit for forensic DNA bones extraction - Macherey-Nagel, Duren, Germany). The DNA molecules were quantified in NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific) and considered with good quality when A260/280 = 1.8. The ideal DNA concentration for PCR was 1 ng/μl. In the PCR reaction was used 5 ng of DNA and the Phusion High Fidelity DNA Polymerase enzyme (Thermo Scientific, Waltham, MA, USA). The NucleoSpin gel and PCR clean-up kit was used to PCR product purification (Macherey-Nagel, Duren, Germany), following the manufacturer's instructions.

The DNA sequencing was performed with the BigDye Terminator v3.1 Sequencing Buffer (Applied Biosystems, Austin, TX, USA), using 4 μl of purified PCR product. The reaction with BigDye v3.1 was purified using xTerminator® BigDye™ Purification kit (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions. Capillary electrophoresis was performed on the ABI PRISM 3500 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For quality control, all specimens were sequenced in triplicate using forward and reverse primers, and excluded specimens when did not reach the sequencing pattern of at least 400 bp for both primers. Sequencing analyses were performed using FinchTv (Geospiza Inc.) and for alignment the ClustalX2 2.0 program was used.

2.5. Design of the MAPK7 gene sequence

Several important steps to primers design for making a complete *MAPK7* gene sequencing were followed (Fig. 1). All *MAPK7* genomic sequence described in two major database, NCBI and Ensembl, were aligned using the program ClustalW EBI (www.ebi.ac.uk/Tools/msa/clustalw2). The longest sequence of *MAPK7* gene described in the analyzed databases was found on the website Ensembl (ENSG00000166484) with 7024 base pairs (bp). This sequence includes 1780 bp upstream ATG region. There is no literature that validates this region upstream of ATG region as the *MAPK7* gene promoter region. There is only one reference, among the products of a company, GeneCopoeia (Rockville, MD, USA) (www.genecopoeia.com) that sells two versions of the plasmids with *MAPK7* promoter (HPRM21154 and HPRM11414). The promoter region on the plasmid HPRM21154 has the 5′ region longer than ENSG00000166484 sequence beginning. The GeneCopoeia Company informed that the sequence was obtained from the Database of Transcriptional Start Sites - DBTSS (http://dbtss.hgc.jp/). The default option of this software database provides the promoter region as 600 pb upstream of the transcriptional start site, so it was selected 760 pb upstream the ATG region described on the ENSG00000166484, to obtain reliable sequencing of the entire gene.

In NCBI, the different transcripts/variants (identified from 1 to 4) result in two different proteins. Variant 1, 3 and 4 give rise to the longer protein with 816 amino acids. On the other hand, the transcriptional start of the variant 2 does not include the original ATG region, giving rise to a truncated protein in its amino-terminal region that is 139 amino acids shorter than the other variants, with only 677 amino acids (Fig. 1).

Thus, the sequence denominated MAPK7 7831 pb includes the promoter sequences described by the GeneCopoeia Company (HPRM21154 and HPRM11414) and the ENSG00000166484 sequence of the Ensembl, including coding regions, introns and 5′ and 3′ regions that flank *MAPK7* gene, which presents further parts of *B9D1* and *MFAP4* genes, respectively. The MAPK7 7831 pb sequence was aligned with chromosome 17, mapping this sequence in the region 19376361-19384171 (NC_000017.11 Reference GRCh 38.p7 Primary Assembly).

The Primer3 program for multiple primers was used to PCR primers design (http://frodo.wi.mit.edu/). This program designed 15 primer pairs overlapping the entire MAPK7 7831 pb sequence (Fig. 1), and each one amplified regions between 500 and 600 bp (compatible with Sanger sequencing). All primer specificity was confirmed using the NCBI blast tool primer (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were also checked regarding known polymorphisms. Primers on the regions with known polymorphisms were avoided or, when it was not possible, primers were constructed with degenerate bases. All forward (F) and reverse (R) primers included the sequence of the universal primer M13, allowing the use of a single primer pair (M13F and M13R) for all sequencing, thus optimizing and simplifying the analysis.

2.6. Statistical analyses

Gene expression analysis was performed using GraphPad Prism 5 software (California). The continuous variables of gene expression were compared using non-parametric tests: *Mann-Whitney*, *Wilcoxon and Kruskal-Wallis*. Categorical variables were performed using the Fisher's exact test. Associations between the SNPs and clinical aspects were estimated by the odds ratio (OR) and 95% confidence interval (CI). The results were considered statistically significant when $P < 0.05$.

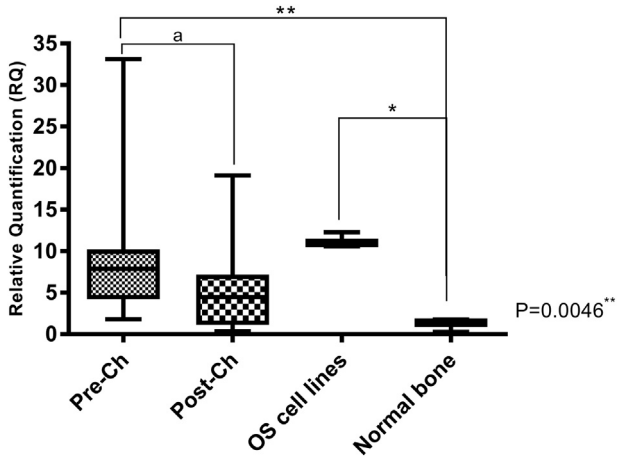

Fig. 2. Quantitative analysis of *MAPK7* gene expression comparing Pre-Ch, Post-Ch, OS Cell lines and normal bone specimens. a - Wilcoxon test showing a statistical tendency between Pre and Post-Ch specimens; Mann-Whitney test *P < 0.05; **P < 0.01.

3. Results

3.1. MAPK7 gene expression in OS

MAPK7 gene expression was performed in 34 frozen tissues specimens and in 3 OS cell lines. We observed difference between 4 groups analyzed (P = 0.0046). We observed *MAPK7* gene expression decrease in Post-Ch specimens compared to Pre-Ch specimens (P = 0.0637). *MAPK7* gene presented overexpression in 100% of Pre-Ch specimens and cell lines in relation to the median of normal bone (P = 0.0025 and P = 0.0171, respectively). (Fig. 2). It is possible to observe that OS gene expression does not have homogeneity distribution; this characteristic has already been described by several papers, which classifies OS in an extremely heterogeneous tumors group [6,10-16].

3.2. MAPK7 gene sequencing in OS

The DNA genomic sequencing was performed to assess if there is any SNP, mutation, deletion or insertion in *MAPK7* gene, which may be responsible for the *MAPK7* gene expression variation in OS Pre-Ch and Post-Ch specimens. All specimens (15 Pre-Ch, 15 Post-Ch, 3 OS cell lines and 4 normal bones) were sequencing for the 15 primer pairs covering the entire *MAPK7* gene. Five SNPs previously described in literature (rs75234643, rs3866958, rs2233072, rs189867712, and rs1054206) and two single nucleotide substitutions, not reported in the literature yet (location on chromosome 17 - loc19381118 A/C and loc19382572 T/G), were found in OS specimens (Fig. 3).

Some specimens presented different genotypes after chemotherapy. The SNPs rs189867712 and rs1054206 were observed in one sample each with this feature. The single nucleotide substitutions, loc19381118, presented in four specimens, and loc19382572, presented in three specimens, also changed the genotype post-chemotherapy. The Hardy-Weinberg Equilibrium was analyzed and only rs1054206 in Post-Ch specimens was not in Hardy-Weinberg Equilibrium (P = 0.0239) (Table 1).

3.3. Correlation between SNPs, pathological clinical data and MAPK7 gene expression

The clinical parameters analyzed are described in Table 2. The detected SNPs were correlated with patient's clinical parameters. A propency significance was observed, in Pre-Ch specimens, between rs2233072G variant allele and metastasis at diagnosis (P = 0.0900) and relapse (P = 0.0455*) (Fig. 4a and b, respectively). In Post-Ch specimens, the rs1054206GG genotype was correlated with osteoblastic histological type (P = 0.0249*) and decrease *MAPK7* gene expression, when compared to rs1054206GC/CC genotypes (P = 0.0095**) (Fig. 4c and d, respectively). No significant association was observed between *MAPK7* SNPs found in OS specimens neither with survival curves nor when compared to normal bone (Supplementary material 1 and 2).

4. Discussion

Progression related to improved survival of OS patients reached a plateau, since the therapy intensification or incorporation of new therapeutic agents has been unsuccessful. In OS, there is no molecular marker that could help in the patient's prognosis and/or treatment, especially in those patients metastatic at diagnosis. Many aspects regarding its pathogenesis remains unknown and questions about the genetic determinants for its development are still unanswered.

Our studies have focused on understanding of the OS biology, especially regarding the interaction of MAPKs pathway with tumorigenesis and drug response. Previous studies of our group have shown that *MAPK7* gene may be a potential biomarker in OS, since patients with *MAPK7* gene overexpression has a worse prognosis [6]. In another study, *MAPK7* gene silencing decreases proliferation, invasion and cellular migration, thus its combined with chemotherapy currently used in the OS treatment may be a promising therapy for this tumor [7]. In the present study, *MAPK7* gene expression levels detected using the TaqMan methodology, corroborated with those found in a previous study that gene expression was assessed by SYBR Green methodology [6] (Applied Biosystems, Inc., Foster City, CA).

Through these findings, our eyes have returned to the individuality of each patient in order to observe if *MAPK7* gene overexpression was linked to a SNP, mutation, insertion or deletion in the tumor genome, and still if the chemotherapy was responsible for difference in *MAPK7* gene expression. There is no cancer study that evaluated the entire sequencing or chemotherapy effect on *MAPK7* gene. This is the first study to investigate the *MAPK7* gene sequencing in OS pre and post chemotherapy specimens. Thus, the present investigation revealed that the rs2233072G variant allele in Pre-Ch specimens was significantly associated with nonmetastatic at diagnosis and relapse absence, suggesting that this variant may be a promising good prognostic marker in OS patients.

The rs1054206 was firstly mentioned in a region without gene described, but currently this region belongs to *MFAP4* gene, a Microfibrillar-associated protein 4 localized in extracellular matrix fibers [17,18] (Fig. 5). The rs1054206GG genotype was correlated with osteoblastic histologic type and downregulation of *MAPK7* gene expression after chemotherapy. In a previous study of our group, we observed that OS cell lines exposed to chemotherapy significantly decreased the *MAPK7* gene expression [7]. All cell lines investigated in the present work presented the rs1054206GG genotype and, interestingly all of them were related to decrease of *MAPK7* gene expression. In literature, there is not any work correlating *MFAP4* and *MAPK7* genes, however, the statistical correlation between rs1054206GG and downregulation of *MAPK7* gene expression in Post-Ch specimens suggested that this SNP could be performing an important role in *MAPK7*, influencing their expression.

Landau et al. [19] found that chemotherapy induced a shift in the clonal composition of the relapsed disease in 10 of 12 treated cases, whereas only one of six untreated cases showed a shift in the clonal composition at relapse. In glioma, the oncogene BRAF V600E, was found in an initial tumor, but was not detected in the recurrent tumor after treatment with chemotherapy (temozolomide) [20]. Bhuvaneshwar et al. (2019) demonstrated novel SNPs and haplotypes in genes associated with drug resistance in OS [21]. In the present study, it was observed that some tumor specimens showed different genotype after chemotherapy. This data suggest that chemotherapy could modify genotypes in *MAPK7* gene and this alteration might be responsible for

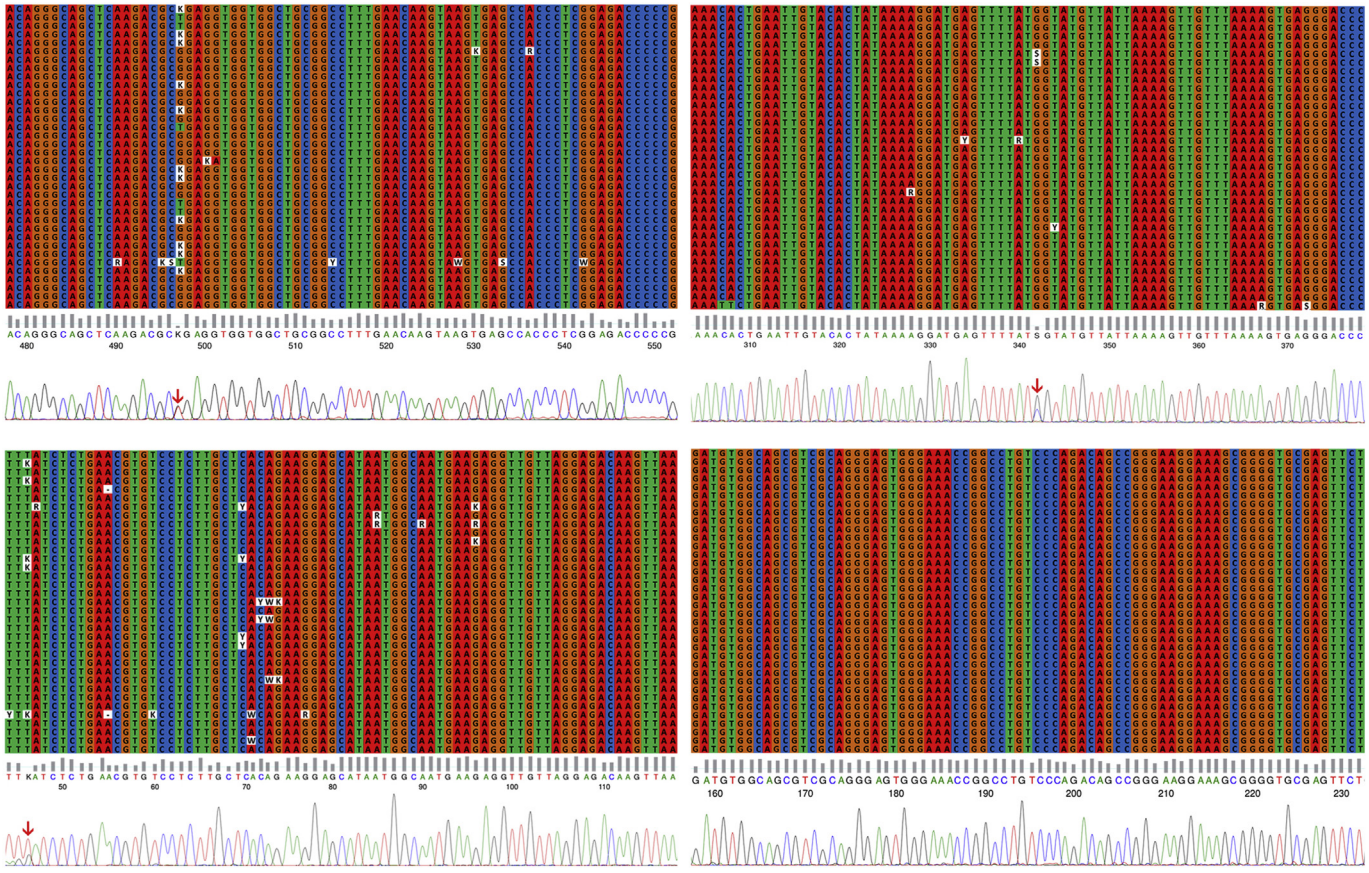

Fig. 3. Sequences alignment from 37 specimens using the ClustalX2 program and chromatogram of a sample illustrating the SNP found using FinchTV program. a) SNP rs 2233072G > T; b) SNP rs75234643G > C; c) example of single nucleotide substitution found not described in the literature, location on chromosome 17 loc19382572T > G; d) complete sequencing without SNP found.

MAPK7 gene downregulation in Post-Ch specimens, contributing to a better treatment response. Our hypothesis is that this genotype exchange could be influenced by chemotherapy or representing a clone selection after chemotherapy, suggesting an acquired or *de novo* mutation [22], once rs1054206GG genotype in Post-Ch specimens is not in Hardy-Weinberg Equilibrium (Table 1).

Qiu et al. [23] found that the rs3866958G variant allele in *MAPK7* promoter was significantly associated with increase of lung cancer risk,

Table 1
Distribution of genotypes found in MAPK7 7381 pb sequence. Only the allele rs1054206, in Post-Ch samples, was not in Hardy-Weinberg Equilibrium.

Polymorphism	Allele	Samples	Genotypes frequencies			Allele frequencies (%)		χ^2; gf	P	HWE
rs75234643			GG	GC	CC	G	C			
	G/C	Pre-CH	14	1	0	96.67	3.33	0.0178; 1	0.8938	Yes
		Post-CH	14	1	0	96.67	3.33	0.0178; 1	0.8938	Yes
rs3866958			CC	AC	AA	C	A			
	C/A	Pre-CH	12	2	1	86.67	13.33	2.685; 1	0.1013	Yes
		Post-CH	12	2	0	92.86	7.14	0.0828; 1	0.7735	Yes
rs2233072			GG	GT	TT	G	T			
	G/T	Pre-CH	6	4	2	66.67	33.33	0.75; 1	0.3865	Yes
		Post-CH	7	5	2	67.86	32.14	0.460; 1	0.4976	Yes
rs189867712			CC	CT	TT	C	T			
	C/T	Pre-CH	11	2	0	92.31	7.69	0.0903; 1	0.7638	Yes
		Post-CH	14	1	0	96.67	3.33	0.0178; 1	0.8938	Yes
rs1054206			GG	GC	CC	G	C			
	G/C	Pre-CH	10	3	1	82.14	17.86	1.017; 1	0.3132	Yes
		Post-CH	11	2	2	80	20	5.104; 1	**0.0239***	No
loc19381118			AA	AC	CC	A	C			
	A/C	Pre-CH	10	4	0	85.71	14.29	0.3889; 1	0.5329	Yes
		Post-CH	15	0	0	100	0	___	0.5329	Yes
loc19382572			TT	GT	GG	T	G			
	T/G	Pre-CH	12	1	0	96.15	3.85	0.0208; 1	0.8853	Yes
		Post-CH	12	3	0	90	10	0.1852; 1	0.667	Yes

The genotyping failed in some samples after three attempts using forward and reverse primer; χ2: chi-square values; HWE: Hardy-Weinberg Equilibrium; df: degree of freedom.

* $P < 0.05$.

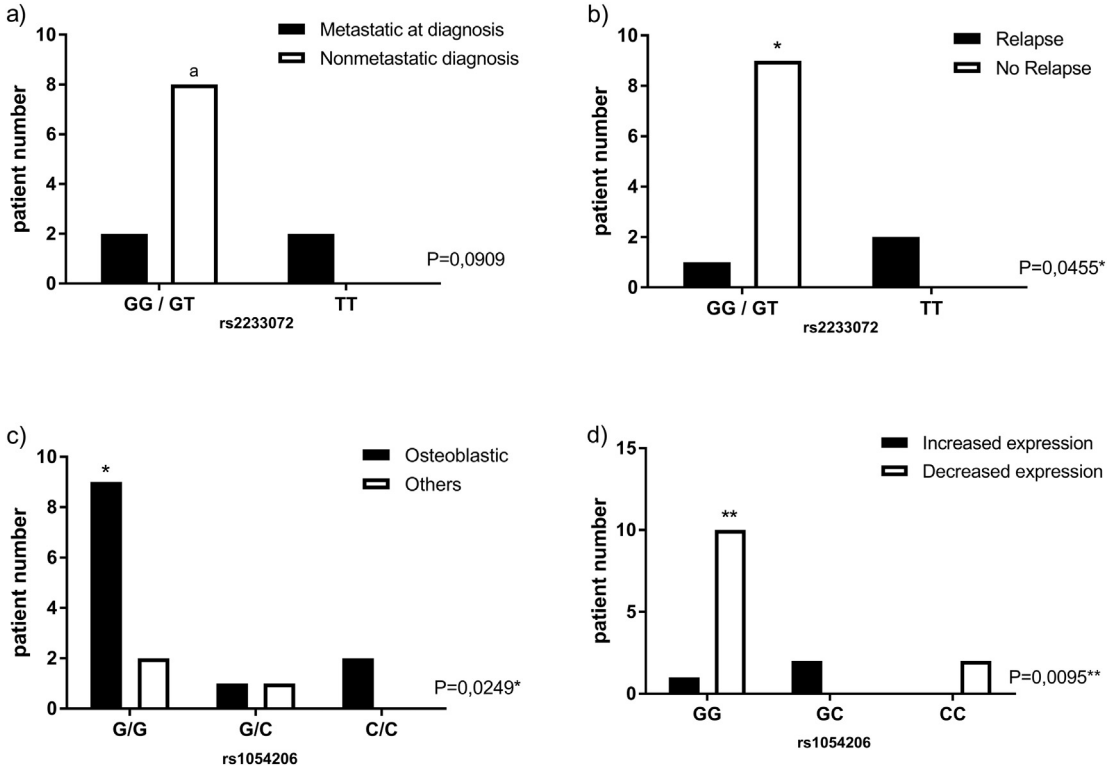

Fig. 4. Fisher test comparing genotypes and clinical parameters. a) rs2233072G variant allele in Pre-Ch specimens, association with nonmetastatic patients; b) rs2233072G variant allele in Pre-Ch specimens, association with no relapse occurrence; c) rs1054206GG genotype in Post-Ch specimens, association with osteoblastic histological type; d) rs1054206GG genotype in Post-Ch specimens, association with *MAPK7* decrease expression.

Table 2
Clinical features of OS patients.

Clinical features	No. (%)
Metastasis at diagnosis	
Yes	9 (60)
No	6 (40)
Tumor location	
Femur	10 (67)
Tibia	3 (20)
Humerus	2 (13)
Tumor size	
≤12 cm	10 (67)
> 12 cm	5 (33)
Histological type	
Osteoblastic	12 (80)
Mixed	1 (6,66)
Chondroblastic	1 (6,66)
Telangiectasic	1 (6,66)
Necrosis grade	
≥90%	8 (53)
< 90%	7 (47)
Type of surgery	
Conservative	13 (87)
Amputation	2 (13)
Relapse	
Yes	4 (27)
No	11 (73)
Status	
Alive	10 (67)
Dead	5 (33)

due to rs3866958G variant allele could enhance the promoter activity, resulting in *MAPK7* overexpression. This SNP was found in three specimens, but it was not correlated with *MAPK7* gene overexpression.

MAPK pathway plays an important role in several cellular processes, mediating the effects of oncogenes range and it has gained great visibility in several cancers. All MAPKs have a very similar structure, which makes it very difficult to use therapy target. Since without a specific target, the chance of causing an even greater lack of control in such an important pathway could not justify the use of therapy target. However, the ERK5, protein encoded by *MAPK7* gene, presents a peculiarity that encourages the development of this therapy type, the presence of a transactivation domain protein that differentiates ERK5 from the other proteins encoded by *MAPKs*. Therefore, we proposed that *MAPK7* plays an important role in OS tumorigenesis, is related to treatment response, prognosis and is a promising therapeutic target.

5. Conclusion

There is a need to identify the poor responders at time of initial diagnosis to avoid delivering ineffective pre-operative therapy. In this study, the rs2233072G variant allele was associated with nonmetastatic disease at diagnosis and relapse absence, and the rs1054206GG genotype was associated with *MAPK7* gene expression downregulation after chemotherapy. The mechanism involved in how *MAPK7* gene affects the tumor behavior has been investigated in a few published papers, but neither of them in bone tumor and especially in paired specimens pre and post chemotherapy. The present study showed that structural changes of *MAPK7* gene was related to prognosis and outcome in OS patients. These findings contributed to recognize that *MAPK* signaling is important in OS tumorigenesis, and that the relationship with chemotherapy response could help to searching strategies for the treatment of OS resistant to current treatment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anndiagpath.2020.151482.

Funding

This work was supported by FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo [grant numbers 2010/10782-8 and

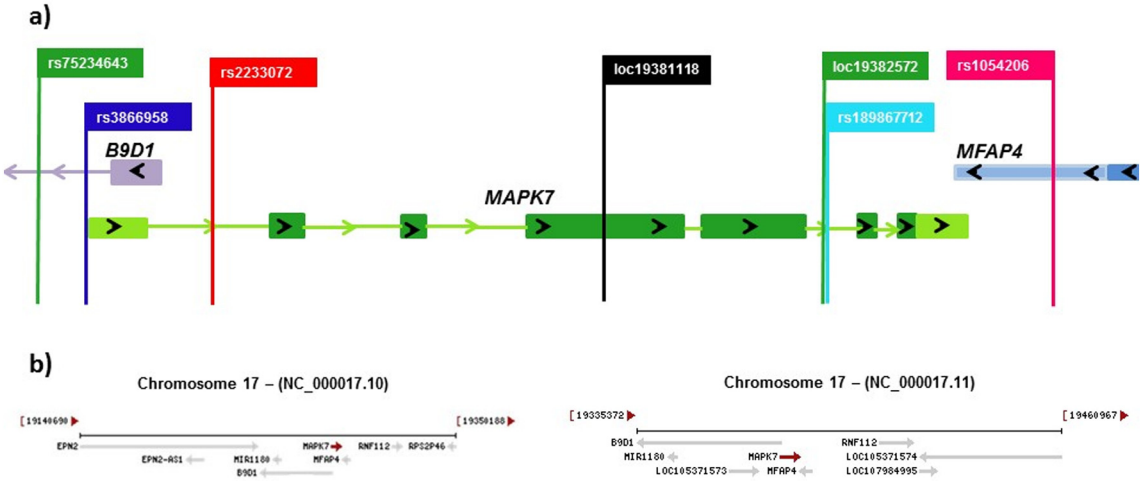

Fig. 5. a) Location of SNPs found; b) Genes structure in two literature moments. NC_000017.10 represents genes organization when the MAPK7 7831 pb was designed and NC_000017.11 represents actual genes organization.

2011/10459-5]; and GRAACC – Grupo de Apoio ao Adolescente e à Criança com Câncer.

Declaration of competing interest

The authors declare that there is no conflict of interest that could constitute an impediment to the publication of this article.

Acknowledgements

We are thankful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2010/10782-8 and 2011/10459-5) and Grupo de Apoio ao Adolescente e à Criança com Câncer (GRAACC/UNIFESP).

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