

RESEARCH ARTICLE

New formulated “DOX-MTX-loaded Nanoparticles” Down-regulate HER2 Gene Expression and Improve the Clinical Outcome in OSCCs Model in Rat: the Effect of IV and Oral Modalities

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Abstract

Background: Oral squamous cell carcinoma (OSCC) remains as one of the most difficult malignancies to control because of its high propensity for local invasion and cervical lymph node dissemination. In this study, we evaluate the efficacy of our novel pH and temperature sensitive doxorubicin-methotrexate-loaded nanoparticles (DOX-MTX NP) in affecting HER2 expression profile in OSCC model in rat. **Results:** DOX-MTX- nanoparticle complexes caused significant decrease in mRNA level of HER2 compared to untreated cancers ($p < 0.05$) and this finding was more pronounced with the IV mode ($p < 0.000$). Surprisingly, HER2 mRNA was not affected in DOX treated as compared to the control group ($p > 0.05$). On the other hand, in the DOX-MTX NP treated group, fewer tumors characterized with advanced stage and decreased HER2 paralleled improved clinical outcome ($P < 0.05$). Moreover, the effectiveness of the oral route in the group treated with nanodrug accounted for the enhanced bioavailability of nanoparticulated DOX-MTX compared to free DOX. Furthermore, there was no significant difference in mRNA level of HER2 ($p > 0.05$). **Conclusions:** Influence of HER2 gene expression is a new feature and mechanism of action observed only in dual action DOX-MTX-NPs treated groups. Down-regulation of HER2 mRNA as a promising marker and prognosticator of OSCC adds to the cytotoxic benefits of DOX in its new formulation. Both oral and IV application of this nanodrug could be used, with no preferences in term of their safety or toxicity. As HER2 is expressed abundantly by a wide spectrum of tumors, i DOX-MTX NPs may be useful for a wide-spectrum of lesions. However, molecular mechanisms underlying HER2 down regulation induced by DOX-MTX NPs remain to be addressed.

Keywords: HER2 - DOX-MTX-NPs - oral squamous cell carcinoma(OSCC) - oral and IV route

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Introduction

Oral squamous cells carcinomas (OSCCs) accounts for approximately 95% of all oral malignant neoplasms and for about 38% of all malignant head and neck tumors, especially the tongue and lip (Jones et al., 1992; Brandwein-Gensler et al., 2005; Bell et al., 2007). Unfortunately, the increase in incidence has not been paralleled by the development of new therapeutic agents and the 5-year survival rate remains at 50% over the past 30 years (Mesgari Abbasi et al., 2014c). Among oral neoplasm, tongue carcinoma exhibit a late diagnosis and a more aggressive form and unfortunately an unfavorable prognosis, the vast majority of Stages III and IV cases are fatal (Schliephake, 2003; Massano et al., 2006; Montoro et al., 2008).

The epidermal growth factor receptor (EGFR)-related

family of receptor tyrosine kinases includes human epidermal growth factor receptor (HER1), EGFR, or c-erbB1; HER2 or c-erbB2 known as her2^{neu}; HER3 or c-erbB3; and HER4 or c-erbB4 (Li et al., 1992). HER2 is a widely studied oncogene in Head and Neck Squamous Cells Carcinomas (HNSCC) and is well prognosticator of disease (Baykara et al., 2013). This tyrosine kinase receptor is connected to various downstream signaling targets involved in cellular proliferation, apoptosis, angiogenesis, invasion, and also metastasis. HER2 encoded by ERBB2 gene and is overexpressed in over 80% of all HNSCC (Jones et al., 1992; Kim et al., 2001; Lippman and Hong, 2001; Kademani et al., 2005; Montoro et al., 2008; Sardari et al., 2012; Khademi et al., 2013).

Combination chemotherapy and nanoparticle drug delivery put forwards significant promise in cancer treatment. Concomitant use of two or more drugs results

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in additive/synergic cytotoxic on cancer cells and can overcome drug resistance through distinct mechanisms of action (Hu et al., 2010; Rossi et al., 2010). On the other side, nanoparticle drug delivery enhances therapeutic effectiveness whilst reduces toxicity on healthy cells through improving their pharmacokinetics. Current advances in improving the efficacy of cancer therapeutics are due to combination of these two dynamic research fields. (Yoo and Park, 2004; Kalaria et al., 2009; Bae, 2010; Wang et al., 2010; Benival and PV, 2012; Deng and Zhang, 2013; Duong and Yung, 2013).

In its unchanged form, doxorubicin has shown substantial treatment potential, being regarded as one of the most potent of the FDA approved chemotherapeutic Drug, limited only by its unspecific toxic effects on healthy cells. However, combined to nanodelivery systems, DOX-nanoparticles not only increase intracellular uptake of DOX, at the same time reduce its side effects significantly compared with conventional DOX formulations (Wang et al., 2010; Tacar et al., 2013).

Methotrexate (MTX) is another central chemotherapeutic drug that is widely used either in monotherapy or in combination with other biologic and synthetic disease modifying anti cancer drugs (Rossi et al., 2010; Cipriani et al., 2014).

DOX- MTX NP is a new combination chemotherapy and nanoparticle drug delivery system that has showed initial promising results in affecting the OSCC in rat model. However more studies require evaluating its efficacy, safety and also the mechanism of action.

In this respect, this study conducted to evaluate the efficacy of IV and oral mode of DOX-MTX-loaded nanoparticles in term of their potential to affect the expression level of Her2/neu (erb\b2) compared to free DOX as a new combination chemotherapy and nanoparticle drug delivery system for treatment of aggressive stages of oral cancer.

Materials and Methods

Dual anticancer drug loaded nanoparticles

The synthesis procedure of nanoparticles was fully explained by Salehi et al (2014). Briefly, appropriate amount of novel synthesized nanoparticles were ultrasonically dispersed in the MTX solution for 5 min. After stirring for 24h under dark conditions DOX-HCl was added to MTX-loaded nanoparticles mixture and dispersed with the aid of ultrasonication (Sonics Vibra cell, Model: VCX 130 PB, Newton, CT) for 3min.

The final carrier/drug ratio was 5 to 1 for both of drugs. The mixture was kept under magnetic stirring at room temperature for another 24h under dark conditions. Then MTX-DOX-loaded nanoparticles dispersion was left for 2 h to allow the sedimentation of the fine precipitates. DOX-MTX-loaded nanocomposites were collected by centrifugation at 14000 rpm for 15min and vacuum dried for 24h at room temperature and stored in a desiccators until used. The dual anticancer drug loaded nanoparticles were diluted with physiologic saline solution in appropriate concentrations before administration to the rats.

Animals

120 male Sprague-Dawley rats weighing 180±20 grams were randomly divided into 8 groups. The animals were housed in the polycarbonate standard cages in a temperature-controlled animal room (22±2°C) with a 12/12 hours light/dark cycle during the experiments. The animals were provided by a standard rat pellet diet *ad libitum*. Drinking water containing 4-NQO was prepared three times a week by dissolving the carcinogen in distilled water and was given in light-opaque bottles.

Establishment of oral squamous cell carcinoma (OSCC) model in rat

OSCC carcinogenesis usually develops through a multistep process that begins from hyperplasia and passes to mild, moderate and severe dysplasia before OSSC. 4-NQO induced OSCC have been used to study the various stages of oral carcinogenesis, because of its capability of inducing sequentially the phases of carcinogenesis (hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ and OSSC). We have previously verified that 4-NQO successfully induces different stages of tongue carcinogenesis process in all cancer groups. High mortality rate, low weight gain, and frequency of OSCC and high proliferation severity of cancer control group compared to other groups demonstrate the efficacy of 4-NQO induced OSCC model (Mehdipour et al., 2013).

Experimental design

120 rats randomly were divided into 8 groups of 15 animals each, as following: (1) Group I served as a carcinoma control and received 4-NQO (Sigma) at the concentration of 30ppm in their drinking water for 14 weeks without any treatment. (2) Group II-III served as the treatment groups and received 4-NQO at the concentration of 30ppm in their drinking water for 14 weeks and oral doses (Gavage) of Doxorubicin and the DOX-MTX-loaded nanoparticles respectively at the dose 5mg/kg of body weight once a day on the days of 2, 5 and 8 of the study. (3) Group IV-V served as the treatment groups and received 4-NQO at the concentration of 30ppm in their drinking water for 14 weeks and intravascular (IV) dosages of doxorubicin and the DOX-MTX-loaded nanoparticles at the dose 1.5 mg/kg of body weight once a day on the days of 2, 5 and 8 of the study. (4) Group VI and VII served as the treated control group that received oral and IV the dose DOX-MTX-NPs (5mg/kg and 1.5mg/kg of body weight once a day on the days of 2, 5 and 8 of the study, respectively). (5) Group VIII served as normal control group and the rats of this group didn't get any carcinogen or treatment material. (6) Death rate of the animals was also recorded during the study.

Ethics

All the ethical and the humanity considerations were performed according to the Helsinki humanity research declaration during the experiments and the euthanasia of the animals. All the animals' experiments were approved by the Ethics Committee of the Tabriz University of Medical Sciences.

Histological evaluations

At the end of the interventional period, the animals were euthanized under anesthetic condition (Pentobarbital, 150mg/kg IP). The tongue tissue samples were taken from each animal and were immediately fixed in 10% phosphate-buffered formalin. The 5µm thick microscopic sections were prepared after embedding of tissue samples in paraffin. Afterward, the sections were stained by Hematoxylin-Eosin staining method and histological evaluations were performed with light microscopy. Histopathological changes in tumors evaluated blindly by two pathologists.

Detection of mRNA expression of HER2 using quantitative real time PCR

Briefly, total RNA (2µg) extracted from homogenized fine powder of removed tongue tissues as described in detail by our group (Jahanban Esfahlan et al., 2011a; 2011b; Jahanban Esfahlan et al., 2012). RNA were reverse transcribed to cDNA using Revert Aid first strand cDNA synthesis kit (fermentase). The resulting cDNA was diluted 1:30 fold and the PCR reaction was performed with 2.5µl cDNA, 10pM each forward and reverse primers, 12µl SYBR Green PCR Master Mix (Fermentase) in a final volume of 25µl. The thermal profile for the real-time Q-PCR was 95°C for 10min and followed by 45 cycles of 95°C for 15sec and 60°C for 1min. The gene expression was expressed as fold change from the GAPDH level which is calculated as $2^{-\Delta\Delta Ct}$. In addition, melting curve analysis was performed to assure the specificity of PCR product in this experiment. The following rat primers were used: ERBB2 (NM_017003.2): TCTCCGTGACCTCAGTGTCTTC-3' (forward), 5'-GTGTCAATGAGTACGCGCCATC-3' (reverse) ; GAPDH (AF 106860): 5'-ATGACTCTACCCACGGCAAG-3' (forward), 5'-CTGGAAGATGGTGATGGGTT-3' (reverse).

Data analysis

The data were analyzed by SPSS 13. One-Way Analysis Of Variance (ANOVA) was used to compare fold change differences of HER-2 between and within studied groups followed by the multiple comparisons with the Tukey post-hoc test. Fischer's exact test used for analyzing pathological changes in groups. Chi square test used to verify the possible relation between expression of HER-2 gene and pathological changes in tissue samples. A p value < 0.05 was considered significant.

Results

Effect of DOX-MTX NP on mRNA expression of HER2 in tongue tissues of OSCC rats models

Our results showed that compared to all three healthy group, HER2 expressed approximately 6.7 fold more in cancerous group ($p=0.000$) (Figure 1). In rats that received oral doses of DOX and DOX-MTX NP (5 mg/kg of body weight once a day on the days of 2, 5 and 8 of the study), results showed that compared to untreated cancerous group, mRNA expression of HER2 decreased 1.6 and 5 fold in DOX ($p=0.075$) and DOX-MTX ($p=0.01$) Figure 2

shows the observed fold change in 5 studied groups (fold changes represented as mean±SE).

Similar results achieved by the IV route. Results showed that after IV treatment with DOX and DOX-MTX NP (1.5 mg/kg of body weight once a day on the days of 2, 5 and 8 of the study), mRNA expression of HER2 decreased 1.6 and 5 fold respectively compared to untreated cancerous that was statistically significant ($p=0.001$), nonetheless in DOX treated group, there was no significant decrease in HER2 mRNA level ($p=0.073$) (Figure 3).

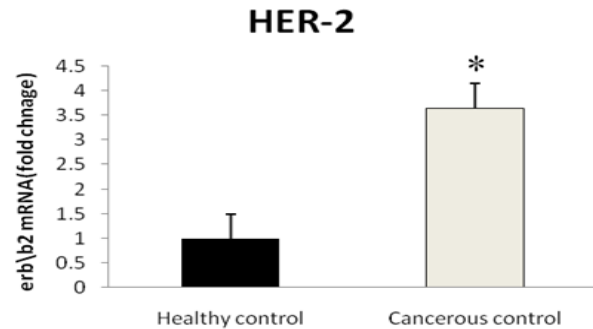


Figure 1. HER2 mRNA Expression in OSCC Cancerous Group and Healthy Control. Compared to healthy group, HER2 over-expressed ~4 fold in untreated cancerous group

* Indicates to a significant p value ($p<0.001$)

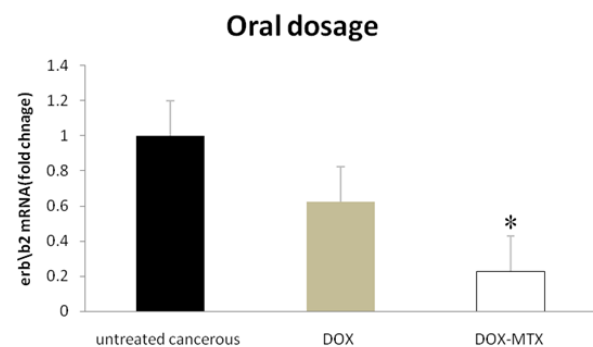


Figure 2. The Effect of Oral Dosage of DOX and DOX-MTX NP on mRNA Level of HER2 in OSCC Cancer Model in Rat. HER2 mRNA level decreased significantly in DOX-MTX NPs compared to untreated cancerous group whilst oral dosages of DOX could not alter amount of HER-2 in treated group *Indicates to a significant p value ($p<0.05$)

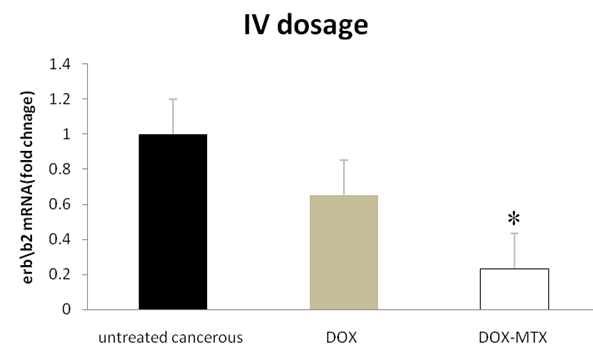


Figure 3. The Effect of IV Dosage of DOX and DOX-MTX NP on mRNA level of HER2 in OSCC Cancer Model in Rat. HER2 mRNA level decreased significantly in DOX-MTX NPs compared to untreated cancerous group whilst IV dosages of DOX could not alter amount of HER-2 in treated group *Indicate to a significant P value ($P<0.001$)

Table 1. Characteristic of Studied Animals in Each Group

| Route of drug administration | Groups classification | Groups names | Treatment | Number of cases(N) (Beginning of the study) | Number of cases (N) (End of study) |
|------------------------------|-----------------------|-------------------|-------------|---|------------------------------------|
| - | I | Cancerous control | - | 15 | 11 |
| Oral (5mg/kg body weight) | II-III | Cancerous groups | DOX | 15 | 13 |
| | | | DOX-MTX NPs | 15 | 14 |
| | | | DOX-MTX NPs | 15 | 15 |
| IV (1.5mg/kg body weight) | V-VI | Cancerous groups | DOX | 15 | 12 |
| | | | DOX-MTX NPs | 15 | 14 |
| | | | DOX-MTX NPs | 15 | 11 |
| - | VII | Healthy control | - | 15 | 15 |
| - | VIII | Healthy control | - | 15 | 15 |

Table 2. Comparison between the Efficacy of IV and Oral Administration of DOX-MTX NP in Lowering the erbB2 mRNA Expression in OSCC Model in Rat

| DOX-MTX NP | | HER2 mRNA | | p-value |
|--------------------|------------|--------------|----------|---------|
| | | Low/moderate | high | |
| pathologic changes | Low stage | 10(90.9%) | 1(9.1%) | p=0.038 |
| | High stage | 0(0%) | 2(100%) | |
| | Total | 10(76.9%) | 3(23.1%) | |

In addition, we find no significant difference between IV and oral administration of DOX-MTX NP (p=0.985) and neither DOX (p=0.87) (Figure 4).

All three healthy controls showed significant difference in HER2 mRNA expression compared to untreated cancerous group (p>0.01). In order to test the **Oral vs IV**

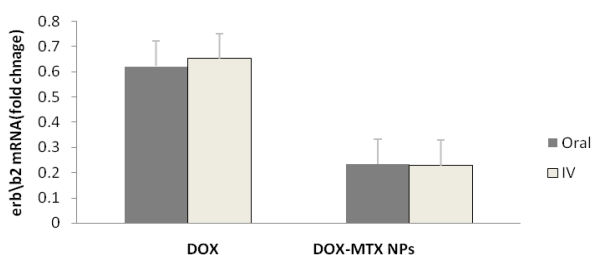


Figure 4. Comparison Between the Efficacy of IV and Oral Administration of DOX-MTX NP in Affecting the HER2 mRNA Expression in OSCC Model in Rat.

In both DOX and DOX-MTX NPs treated groups, there was no difference between IV and oral route in decreasing the amount of HER2 mRNA level in OSCC model in rat. *Indicates to a significant p value (p<0.05)

Healthy groups

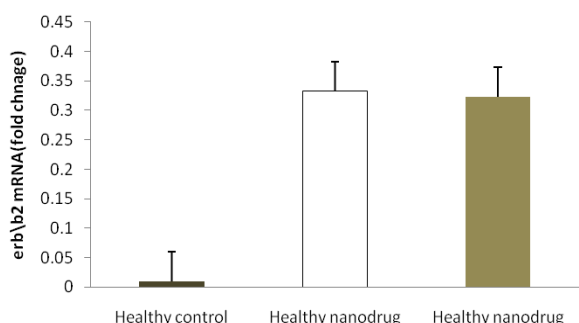


Figure 5. The Effect of Oral and IV Dosage of DOX-MTX NP on mRNA Level of HER2 in Healthy Rats.

There was no significant difference in HER-2 amount of treated healthy in both modalities of nanodrug with that of untreated healthy control. *Indicates to a significant p value (p<0.05)

tumor specificity and also safety of our nanodrug safety on normal cells, we have used two healthy groups treated with the same doses of our nanodrug (Figure 5). Result showed that both IV and oral healthy controls showed no significant difference in HER2 expression compared to untreated healthy control (p=0.93).

Furthermore, our result indicate that both IV and oral administration of MTX-DOX has superior activity (~3 fold) over free DOX in down-regulating the expression of HER2 in OSCC model in rat (p=0.014 and p=0.045, respectively) (Figure 6).

Histopathological changes in DOX and DOX-MTX NP treated groups

AS IV mode of nanodrug showed superior performance over oral form, therefore this group subjected for evaluation of histopathological changes.

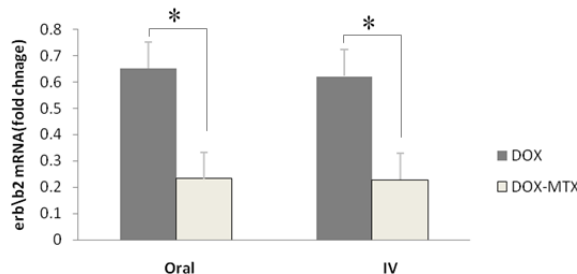


Figure 6. Comparison between the Efficacy of Oral and IV Dosage of DOX and DOX-MTX NP in Affecting the HER2 mRNA Expression in OSCC Model in Rat.

In both DOX and DOX-MTX NPs treated groups, IV modality exhibit superior performance over oral route in decreasing the amount of HER-2 mRNA level in OSCC model in rat. *Indicate to a significant P value (P<0.05)

IV dosage

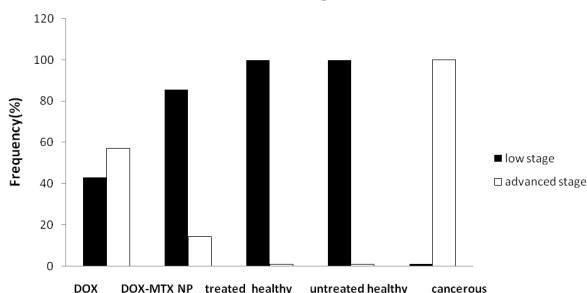


Figure 7. Frequency of Observed Histopathological Changes in DOX and DOX-MTX NPs Treated Groups (IV route)

Our results showed that in DOX treated group 6/13 of lesion showed a low stage (No/Mild/moderate dysplasia) while 7/13 were advanced (Severe dysplasia, Carcinoma in situ and OSCC) (Figure 4). At the other hand, we observed markedly increase in frequency of low stage tumor (12/14 vs 2/14) in group treated with IV doses of nonodrug. Pathological changes significantly were different between the groups ($p < 0.05$). Furthermore, no pathological changes detected in either of healthy controls, whilst all rats of cancerous group were found to develop aggressive lesions (Figure 7).

Relation between HER2 mRNA with tumor stage in OSCC samples

Subsequently, we tested the possible relation between HER2 mRNA profile and the tumor stage in DOX-MTX NP treated group. In this respect according to the observed mRNA fold changes, samples categorized in two main groups: group with high mRNA and group with low/moderate mRNA level. As shown in Table 2, 10/11 showed low level of HER2 (90.9%) and 1/11 exhibit high amount of HER2 level (9.1%). In high staged group, all of them (2/2) had a high level of HER2 (100%) ($p = 0.038$). It should be noted that HER2 mRNA was not detectable in one of low advanced lesions (Table 2).

Discussion

It is known long time before that by combination of two or even more than single agent therapy can overcome the limited success in cancer therapy due to the toxicity at high drug dosage, the heterogenic tumors and more importantly the acquired drug resistance (Abusail et al., 2013; Lasrado et al., 2014). Hence, combination therapy uses combined regimes that multiply the additive effects to the targeted cancer cells (Tacar et al., 2013). Afterwards, when synergistic combination with enhanced therapeutic effects get combined with an appropriate nanodelivery system such as the nanoparticles, a much powerful anti-cancer weapon create that could efficiently and specifically target tumoral cells (Yoo and Park, 2004; Hu et al., 2010; Wang et al., 2010).

With different activities to cancer cells, the pairing of chemotherapeutic agents doxorubicin (DOX) and Methotrexate (MTX) for combination treatment can have higher and synergistic therapeutic effect. DOX known as a DNA intercalator that inhibit nucleic acid synthesis while MTX is an antimetabolite (Duong and Yung, 2013).

HER 2 belongs to Epidermal Growth factor families and its role extensively investigated. This tyrosine kinase receptor, controls important processes such as apoptosis, cell proliferation and metastasis. Its overexpression well correlated with aggressive and invasive potential in different types of cancers as well as OSCC (Li et al., 1992; Lippman and Hong, 2001). In this view, as HER2 overexpression is restricted to cancerous cells, this feature makes it a validated target for designing drugs.

In this study we evaluate the efficiency and safety of two different forms of our novel stimuli-responsive cationic mesoporous silica nanoparticles (MSNs) loaded

with DOX-MTX in affecting the HER2 mRNA level *in vivo*. Modified MSNs provide a pH and temperature-triggered release of entrapped drugs at tumor tissue environment (lower pH and higher temperature than physiological condition). An efficient anticancer performance of Multi anticancer drug-loaded MSNs previously verified by DAPI staining and MTT assay tests. This formulation provides cooperative thermo and pH-responsive targeted delivery of DOX and MTX to the cancerous tissues (Salehi et al., 2014).

Cytotoxic effect of Doxorubicin most often attributed to its potential in induction of apoptosis by recruiting divergent targets as well as p53 (Gibson et al., 2005; Huang et al., 2011). However to our knowledge there is no record for Doxorubicin induced HER2 mRNA down-regulation in tumors, nonetheless in our study, none of oral and IV forms of DOX influenced the HER2 mRNA level in treated groups, surprisingly both forms of the new formulated DOX (DOX-MTX NP) showed high performance in affecting HER2 mRNA level and interestingly this decrease in HER2 paralleled with less aggressive tumors in this group.

We have previously shown the higher performance of DOX-MTX NP in affecting p53 (as a main tumor suppressor gene) and Matrix Metalloproteinase 2 (MMP-2) (as a important prognosticator for OSCC invasion and metastasis) (data not published), In those studies, although p53 mRNA level was significantly increased in DOX treated group, but however free DOX was not able to affect the MMP-2 mRNA level and neither HER2 as another prognosticator for aggressive tumors (Mesgari Abbasi et al., 2014b; Mesgari Abbasi et al., 2014). Overall, we conclude that as these genes are affected only by DOX-MTX NPs, hence other mechanism of action related to enhanced cytotoxic effects of combinatorial treatment of DOX and MTX should be involved. Further evaluation for revealing the underling mechanism of action for this new feature acquired by novel formulated DOX-MTX NPs could be interesting in part and may introduce this novel nanodeug as a potent multifunctional drug in treatment of a wide range of malignancies.

In conclusion, according to the obtained results, DOX-MTX NPs is a safe drug that has the potential to inhibit activity of HER2 and improve the outcome of disease in invasive stages of OSCC.

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