Molecular Mechanisms of Chemoresistance in Oral Cancer

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Oral cancer is an aggressive disease with the propensity for local recurrence and distal metastasis in the head and neck region. Currently, cisplatin-based chemotherapy or concurrent radiochemotherapy is still the first choice to treat the advanced stage cancers, in particular, the unresectable tumours. Unfortunately, innate and acquired resistance to chemotherapy agent greatly limited its effectiveness and often led to treatment failure in these patients. Hence, it is urgent to clarify the mechanisms underlying the development of chemoresistance in patients with oral cancer. In this article, the current understandings on molecular mechanisms of chemoresistance in oral cancer were reviewed, including drug efflux, apoptosis, DNA damage and repair, epithelial mesenchymal transition, autophagy and miRNA.

Key words: *chemoresistance, molecular mechanisms, oral cancer*
Chin J Dent Res 2016;19(1):25–33; doi: 10.3290/j.cjdr.a35694

Oral cancer is one of the most common malignancies in the head and neck region, with properties of rapid local invasion and high recurrence rate. Generally, the treatment modalities for oral cancers included surgery, radiotherapy, chemotherapy and immunotherapy. Based on current treatment guidelines, early stage cancers of oral cavity are treated with surgery or radiotherapy alone while advanced stage cancers are usually treated with a comprehensive, sequential, multi-modality regime, including surgical resection of primary tumours followed by radiotherapy with or without chemotherapy[1]. Cisplatin-based concurrent radiochemotherapy is still the gold standard for the treatment of unresectable tumours. Recently, several new approaches have also been reported in the treatment of oral cancer, including targeted therapy against epidermal growth factor receptor, induction chemotherapy and p53 gene therapy[2]. Unfortunately, the survival rate and prognosis are still not satisfactory in patients with advanced stage oral cancer. To a certain extent, inherent and acquired resistance to chemotherapy agents contributes to treatment failure in these patients. The development of chemoresistance phenotype greatly limits the effectiveness of chemical agents in oral cancer. Therefore, it is urgent to elucidate the mechanisms underlying the development of chemoresistance in patients with oral cancer. In this article, we will review the current knowledge on the molecular mechanisms of chemoresistance in oral cancer.

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This work was supported by the National Natural Science Foundation of China (81272949, 81202136, 81172567, 81372885 and 81572661), Specialized Research Fund for the Doctoral Program of Higher Education (20120171120068), Pear River S&T Nova Program of Guangzhou (2014J2200045) and Fundamental Research Funds for the Central Universities (11ykpy47).

Drug transporters and chemotherapy resistance in oral cancer

Multi-drug transporters are the transmembrane proteins which are widely known for their contributions to chemoresistance in cancer. Through promoting the efflux of anti-cancer therapeutic agents from tumour cells and decreasing the intracellular level of drugs, they exert an important role in multi-chemoresistance (MDR). The adenosine triphosphate (ATP) binding cassette (ABC) transporter superfamily is the most well documented multi-drug transporters, which has been classified into

seven subfamilies designated with A to G, based on the sequence and structural homology, such as ABCB1 (also known as MDR1 or P-gp), ABCC1 (also known as MRP1), ABCG2 (also known as BCRP or MXR) and so on[3,4].

P-gp, a 170 KDa phosphoglycoprotein, is encoded by the MDR1 gene, which can function as a unidirectional ATP-dependent pump to export drugs from cells. It is normally expressed and plays a physiological role in the renal tube, intestinal epithelium and the placenta trophoblast by excluding metabolic waste, in order to protect the cell from xenobiotics. In cancer cells, the main substrates are origins of plants, including taxanes, alkaloids and podophyllotoxins. Therefore, P-gp positive oral cancer cells may rapidly eliminate paclitaxel and vincristine. The expression of P-gp in oral squamous cell carcinoma (OSCC) was firstly detected by Jain et al with flow cytometry, and they showed that expression of P-gp was increased in recurrent OSCC compared to normal mucosa with oral lesions at different stages of tumorigenesis[5]. Similar results were also confirmed by several immunohistochemical studies[6-8]. Intriguingly, the expression of P-gp could be induced by treatment with therapeutic drugs or radiotherapy in oral cancer cells[9,10], and silencing them with inhibitors or siRNA may enhance the sensitivity for treatment[11,12]. MRP1, a well characterised member of the ABCC subfamily, was firstly reported in 1992 in lung cancer cell lines[13]. Organic anions are the preferable substrates for MRP1, including drugs conjugated to glutathione, glucuronate or sulfate. This protein contributes to MDR for etoposide, antracyclic antibiotics, vinca-alkaloids, stibium and arsenium drugs as well as methotrexate. It has been proven that increased expression of MPR1 was detected in OSCC compared with adjacent noncancerous epithelium and this correlated with chemoresistance and poor prognosis in OSCC patients[14]. Naramoto et al showed that enhanced expression of MRP1 was detected in OSCC cell lines treated with vincristine[15]. Similar results were also observed in OSCC cells treated with cisplatin[9]. ABCG2 is another important drug transporter and well known as a marker of side population cells which are enriched with stem cells. Overexpression of ABCG2 was involved in the resistance to mitoxantrone, antracyclic antibiotics (in particular, doxorubicin), methotrexate, camptothecin derivatives (topotecan and SN-38) and indolocarbazole derivatives. In OSCC, it was confirmed that side population cells harboured cancer stem cell properties with a high expression of ABCG2[16,17]. Further studies showed that these SP cells demonstrated a multi-chemoresistance phenotype for 5-FU[18], carboplatin[19], Bortezomib and etoposide[20]. Interestingly, the common molecular characteristic in these SP cells is the increased expression of ABCG2. Recently, Yanamoto et al further demonstrated that local recurrence in OSCC patients treated with neoadjuvant chemotherapy is associated with enhanced expression of ABCG2[21]. Altogether, these findings suggest that multi-drug transporters, including P-gp, MRP1 and ABCG2 may contribute to both inherent and acquired chemoresistance in oral cancer. However, little is known about the regulation of P-gp, MRP1 and ABCG2 involvement in oral cancer.

Dysfunction of apoptosis in the chemoresistance of oral cancer

Increasing evidence suggested that resistance to apoptosis is one of the hallmarks of cancer and leads to treatment failure. It is required that cancer cells are addicted to some anti-apoptotic proteins for survival during the cancer development and progression. Therefore, dyregulation of apoptosis-related genes and pathways are definitely involved in the chemoresistance of cancers. Of these, the Bcl-2 family members, survivin and the p53 pathway are well-documented[22-24]. The Bcl-2 protein family is comprised of anti-apoptotic proteins (e.g. Bcl-2 and Bcl-xL) and pro-apoptotic proteins (e.g. Bax, Bak and Bad). Increased expression of anti-apoptotic proteins (Bcl-2 and Bcl-xL) and decreased expression of pro-apoptotic proteins (Bax) were demonstrated to contribute to oral cancer development[25,26]. Not surprisingly, upregulation of Bcl-2 and Bcl-xL was found in several drug-resistant OSCC cells[27,28]; and silence of Bcl-xL and Bcl-2 was able to enhance chemosensitivity of OSCC cancer cells[29,30]. These data indicated that Bcl-2 family have a pivotal role in chemoresistance and may serve as a potential molecular target in reverse chemoresistance. However, further clinical trials are necessary to validate the effect of targeting the Bcl-2 family to reverse chemoresistance in oral cancer.

Survivin, a member of the inhibitor of the apoptosis protein family, has been identified as one of the most important biomarkers in the determination of chemoresistance. Survivin could block apoptosis to promote cell proliferation and survival through directly binding to caspase, which in turn favour chemoresistance. Enhanced expression of survivin has been observed in OSCC, and may act as a predictor of tumour aggressiveness and poor outcome[31-33]. Further studies indicated that increased expression of survivin may involve the HPV-mediated deregulation during maturation of squamous epithelium through modulation of the apoptotic processes[34]. Interestingly, the

sublocation of survival also has a crucial role for the prognosis in OSCC patients. Engels et al demonstrated that nuclear survivin revealed a favourable prognosis while cytoplasmic survivin indicated an unfavourable outcome and the survivin's cytoplasmic localisation was determined by Crm1-mediated nuclear export[35,36]. Our series studies confirmed that survivin could block caspase-3-dependent apoptosis to enhance cisplatin resistance in OSCC[37-39]. Further studies showed that nicotine could repress cisplatin-induced apoptosis of human oral cancer cells, and depletion of survivin abolished the protective effects[40]. Recently, Sepantronium bromide (YM155), a selective small-molecule survivin-suppressant was developed and demonstrated ideal anti-tumour activity. Kumar et al showed that YM155 could reverse cisplatin resistance in head and neck cancer by decreasing cytoplasmic survivin levels *in vitro* and *in vivo*[41]. YM155 could also trigger apoptosis in a mitochondrial- and death receptor-dependent manner in head and neck squamous cell carcinoma (HNSCC). Moreover, YM155 was found to induce autophagic cell death by enhancing Beclin1 expression. These results indicated survivin-targeted gene therapy may have a dual role in cell apoptosis and autophagy[42].

P53 is the most famous tumour suppressor and the guardian of the genome. Mutation of p53 was frequently observed in most of the human cancers. Mutant p53 correlates with chemoresistance of oral cancer cells due to its inability for inducing apoptosis. *In vivo* studies confirmed that xenografts with p53 mutation showed a significantly higher resistant phenotype to cisplatin[43]. Similar results were also observed in some clinical trials. Temam et al showed that p53 gene mutations were strongly associated with a risk of response to chemotherapy[44]. Cabelguenne et al demonstrated that the prevalence of p53-mutated tumours was higher in the group of patients with nonresponse to neoadjuvant chemotherapy than in the group of responders[45]. Interestingly, in another prospective randomised clinical trial, Perrone et al reported that the loss of function (transactivation activities) of p53 mutant proteins may predict a significantly low pathological complete remission rate in patients with SCC of oral cavities treated with cisplatin-based neoadjuvant chemotherapy[46]. More interestingly, Tonigold et al showed that head and neck squamous cell carcinoma cell lines with cytoplasmically sequestered mutant p53 (p53(mut_c)) are frequently more resistant to cisplatin than cells with mutant but nuclear p53 (p53(mut_n), and revealed that cells with p53(mut_c) were endowed with a stem cell-like phenotype, which was associated with ABCC2 overexpression[47]. These findings indicated that the biological function of p53 has played a pivotal role in the chemosensitivity of oral cancer. Mutations of gene, function and subcellular localisation of p53 protein (mutated and wild-type) are all involved in the chemoresistance of cancer cells through intriguing mechanisms which regulate apoptosis, cell cycle, stemness and DNA damage. Therefore, a much better understanding of the cellular networks involving p53, associated biomarkers, and their relationship to responses to therapy might eventually overcome chemoresistance and enhance sensitivity to chemotherapy in oral cancer.

DNA damage and repair in the chemoresistance of oral cancer

Currently, chemotherapeutic drugs routinely used in the treatment of oral cancer always function to damage DNA, including cisplatin, carboplatin, oxaliplatin and 5-FU. The enhancement of DNA repair capacity is crucial for cisplatin-based chemotherapy. DNA repair are mainly dependent on mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), homologous recombination (HR), translesion DNA synthesis (TLS) and nonhomologous end joining (NHEJ) in normal physical conditions[48]. Of note, the NER system is believed to resolve the majority of DNA lesions provoked by CDDP[49]. It has been reported that alterations of NER-related genes and pathways contributed to cisplatin-resistant phenotypes in oral cancer cells. Amongst these genes, the most important determinant involved in cisplatin resistance is excision repair cross-complimentary group 1 (ERCC1), which is the key components of the NER pathway. The expression of ERCC1 was enhanced in carboplatin-resistant TSCC cell lines compared with its sensitive counterparts[50]. Cisplatin was also suggested to induce increased ERCC1 expression through the regulation of the MAPK signalling[51]. Conversely, silencing of ERCC1 was demonstrated to reverse chemoresistance to cisplatin in gastric carcinoma[52] and ovarian carcinoma[53]. In clinical trials, high expression of ERCC1 was associated with unfavourable overall survival than patients with low levels in HNSCC patients treated with cisplatin-based therapy[54-57]. Importantly, the cisplatin response is modulated by more than just ERCC1 expression levels. Polymorphisms have also been shown to be play an important role in chemoresistance[58]. Yang et al also confirmed that the genotypes of XRCC1 rs1799782 and XRCC2 rs2040639 DNA repair genes appeared to be significantly associated with oral carcinogenesis[59]. Interesting, XRCC1 was also involved in EMT-mediated chemoresistance and Snail could directly enhance the expression of XRCC1 at transcrip-

tion level[60]. On the other hand, NHEJ was also involved in chemoresistance in head and neck cancer. Banerjee et al found that TRIP13 was overexpressed in HNSCC and promoted error-prone NHEJ to induce chemoresistance and provided a novel target for overcoming chemoresistance in HNSCC[61]. Taken together, NER system has a critical role in platinum-based treatment and may serve as a predictor in whether HNSCC patients respond to platinum-based chemotherapy. However, the translation of these findings into the clinical setting has still not been forthcoming and further studies are necessary to clarify the complex mechanism underlying CDPP resistance of oral cancers.

Epithelial mesenchymal transition in chemoresistance of oral cancer

Epithelial mesenchymal transition (EMT) is a dynamic biological process by which epithelial cells lose their cell polarity and adhesion to become mesenchymal-like cells with properties of invasive and migratory ability. It has been demonstrated that genetic and epigenetic alterations were involved in EMT. The decreased expression of E-cadherin is one of the most important hallmarks of EMT. The transcriptional factors seem to be the key regulators to drive EMT procedure by targeting CDH1 promoters, such as Snai1, Snai2, Twist1, ZEB1 and ZEB2. Cytokines have also played a key role in EMT which are abundant in the tumour microenvironment, including TGFβ, EGF, FGF, IL, SDF-1 and so on. These cytokines could induce EMT via a distinct signal pathway and activate EMT-related transcriptional factors. Presently, increasing evidence suggests that there are intricate links between EMT and chemoresistance in cancer. Studies showed that cisplatin, 5-fluorouracil and EGFR inhibitor-resistant cancer cells were endowed with EMT phenotype. Maseki et al reported that gefitinib-resistant cancer cells demonstrated an EMT phenotype through activation of the Akt/GSK-3β/snail signalling pathway[62]. Harada et al confirmed that 5-fluorouracil-resistant cancer cells showed epithelial to mesenchymal transition changes in OSCC[63]. More importantly, Sun et al established stable chemotherapy resistant tongue squamous cell carcinoma (TSCC) cell models with a gradual increase of cisplatin and confirmed that cisplatin-resistant TSCC cells displayed a mesenchymal phenotype compared with their parental cells. Further studies found that reduced expression of miR-200b and miR-15b were involved in chemotherapy induced EMT in TSCC[64]. These findings confirmed that chemotherapy could promote EMT and metastasis in oral cancer, which indicated the residual cancer cells may have a much more aggressive capability after chemotherapy and promote cancer progression. Emerging evidence also indicated multi-chemoresistance could be as a result of EMT. An increased expression of ABC transporters and enrichment of cancer stem cells mainly contributed to EMT-mediated chemoresistance. Our experiments confirmed that Snai2 and EZH2 could inhibit E-cadherin to promote EMT in OSCC[65,66]. Intriguingly, Snail could promote EMT and drive erlotinib resistance in oral cancer[67]. In addition, Snail1 could also induce EMT to promote cancer stem cell-like phenotype in head and neck cancer[68]. Mechanically, the activation of ERCC1 by Snail is critical to the generation of cisplatin resistance in head and neck squamous cell carcinoma, which plays a pivotal role in the process of DNA damage and repair[60]. Based on these findings, it is suggested that chemotherapy may induce EMT to promote invasion and metastasis, which in turn lead to chemoresistance in cancer cells. The feedback loops work together to promote malignant progression of oral cancer. A better understanding of the complexities behind this process may offer the opportunity to modify and develop new chemotherapeutic agents which may serve to improve outcomes in oral cancers.

miRNA dyregulation and resistance to chemotherapy in oral cancer

MicroRNAs (miRNAs) are a class of endogenous, small, non-coding, single-strand RNA molecules which have been confirmed to be involved in a wide range of biological processes in physiological and pathological conditions. Generally, miRNA is originally from the primary miRNA (pri-miRNA), which is transcribed by RNA polymerase II and processed by Drosha and Dicer to produce mature miRNAs. The mature miRNA was assembled to form the RNA-induced silencing complex (RISC) together with the RISC-associated proteins, i.e. members of the Argonaute family. This complex was directed to silence gene expression via binding to the 3'UTR of the target mRNAs with imperfect complementarity[69]. Further studies also found that miRNA could regulate targeted gene expression by binding to the 5'UTR and CDS region[70].

Accumulating studies confirmed that miRNAs played an important role in cancer initiation, progression and chemoresistance. Yu et al firstly reported the differential expression profile in oral cancer using cisplatin-sensitive TSCC cell line (Tca8113) and its cisplatin-resistant subline (Tca/cisplatin). A total of 19 deregulated miRNAs (17 upregulated and two downregulated) were identified in Tca/cisplatin cells compared with Tca8113

cells. Further studies confirmed that silence of miR-213 or miR-23a and the rescue of miR-21 could partially abolish the chemoresistance against cisplatin in Tca/cisplatin cells. These results suggested that miR-21 may serve as a chemosensitive miRNA, while miR-214 and miR-23a serve as chemoresistant miRNAs in TSCC cell lines[71]. However, these findings seem to be cell-specific and cannot be validated by other TSCC cells and clinical samples. A recent study showed that miR-23a promotes cisplatin chemoresistance and protects against cisplatin-induced apoptosis in TSCC cells through regulation of the Twist level[72]. In addition, downregulation of miR-100, miR-130a and miR-197 and upregulation of miR-101, miR-181b, miR-181d and miR-195 expression were demonstrated in HNSCC cells with docetaxel-induced multi-chemoresistance[73]. However, the direct target genes were not identified and validated in these studies mentioned above. To further elucidate the mechanism of miRNAs involved in acquired chemoresistance in oral cancer, an excellent study performed by Sun et al showed that miR-200b and miR-15b were significantly decreased in CDDP-resistant CAL-27 cells. Both miR-200b and miR-15b could reverse cisplatin-induced EMT and enhance chemosensitivity by targeting BMI1 in TSCC64. Our work also found that miR-181 regulated EMT and chemoresistance by targeting Twist1 in CAL27 cells[74]. Similar results were also observed in Let-7d, which may regulate EMT and chemoresistance through the inhibition of Twist and Snail in oral cancer[75]. These findings indicated that dysregulation of miRNAs has a critical role in EMT-mediated chemoresistance in oral cancer.

Moreover, increasing evidence suggested that apoptosis-related miRNAs contributed to chemoresistance. MiR-21, the most famous oncomiRNA, is an independent and poor prognostic factor and functions as an apoptotic inhibitor through the silencing of TPM1 and PTEN[76]. MiR-21 was also involved in the resistance to chemotherapy in oral cancer. Downregulation of miR-21 could sensitise CA-27 cells to cisplatin by increasing cisplatin-induced apoptosis in OSCC[77]. Similar results were also reported by Ren et al who demonstrated that miR-21 could modulate chemosensitivity of TSCC cells to cisplatin by targeting PDCD4[78]. Further experiments confirmed that miR-21 is necessary in Nanog-Stat3 mediated chemoresistance[79]. Furthermore, miRNAs including miR-222[80], miR-375[81] and miR-29a[82] were also shown to regulate chemoresistance by targeting apoptosis. Interestingly, a novel mechanism in which mitochondrial fission participated in the regulation of chemoresistance was reported. These studies demonstrated that miR-483-5p could inhibit mitochondrial fission and cisplatin sensitivity by targeting FIS1[83] and BRCA1-miR-593-5p-MFF axis-mediated mitochondrial fission and apoptosis, which also affected cisplatin sensitivity in TSCC[84].

More recently, emerging evidence also indicated that miRNAs can target stemness in order to regulate chemoresistance in oral cancer. Yang et al showed that let-7a represses chemoresistance by modulating the expression of stemness genes[85]. HA-induced CD44v3 interaction with Oct4-Sox2-Nanog signalling plays a pivotal role in miR-302 production leading to AOF1/AOF2/DNMT1 down-regulation, survival of protein activation and cisplatin resistance in cancer stem cells from HNSCC[86]. These observations suggest that miRNAs mainly contribute to chemoresistance through the regulation of EMT, apoptosis and stemness in oral cancer at post-transcriptional level. Of note, oncogenes and tumour suppressors could also regulate the expression of chemoresistance-related miRNAs directly or indirectly.

Autophagy related chemoresistance in oral cancer

Autophagy is an evolutionary conserved catabolic process where cells self-digest intracellular organelles to regulate normal turnover of organelles and remove damaged organelles with compromised function, in order to further maintain homeostasis. Currently, the role of autophagy in cancer is still controversial. Constitutive autophagy can act as a cellular housekeeper to eliminate damaged organelles and protect cells against carcinogenesis. Autophagy can also function as a pro-survival signal in response to stress such as nutrient deprivation, hypoxia and the presence of chemotherapy or some targeted therapies that might mediate resistance to anticancer therapies in advanced cancer. On the other hand, excess or persistent autophagy is shown to promote cell death by enhancing the induction of apoptosis or mediating 'autophagic cell death'. Therefore, autophagy is a double-edged sword in cancer progression. In oral cancer, Wang et al[87] and Weng et al[88] demonstrated that a decrease of autophagy activity could promote malignant progression of TSCC, and Beclin1 can serve as a tumour suppressor in TSCC development. Conversely, studies also showed that LC3, ATG9a, Beclin1 and ATG5 overexpression were associated with a poor prognosis in patients with OSCC in a series of studies[89-91]. These findings indicated both a decrease and increase of autophagy activity have a crucial role in OSCC progression and we propose that the roles of autophagy may be dependent on cancer type, stage, genetic background and the tumour microenvironment. Similarly to

its potential to either induce cell death or promote cell survival, emerging evidence implicated that autophagy has a dual role in response to chemotherapy in cancer. Inhibition of autophagy can enhance the chemotherapeutic sensitivity of cisplatin in OSCC[92], hypopharyngeal carcinoma[93], salivary adenoid cystic carcinoma[94] and oesophageal squamous cell carcinoma[95]. Moreover, DNA-damaging agents including cisplatin, methotrexate and 5-fluorouracil could induce autophagy with a cytoprotective effect[96,97]. An enhanced expression of Beclin-1, Atg12-Atg5 and LC3-II and autophagosome formation was detected in the methotrexate-resistant SCC-9 cell line compared with the sensitive SCC-9 cell line[98]. Similar findings were observed in laryngeal cancer, in which exposure to cisplatin induced the aggregation of autophagosomes in the cytoplasms and increased expression of Beclin 1 and LC3II, and the induction of autophagy attenuated cytotoxicity of cisplatin treatment[99]. These results implicated that enhancement of autophagy could contribute to chemoresistance in head and neck cancers. Inhibition of autophagy may be a potential target to reverse chemoresistance in cancer treatment. However, it should be noted that autophagic cell death could also be induced in oral cancer cells to promote cell death. Zhang et al found that targeting survivin by YM155 can benefit HNSCC therapy by increasing apoptotic and autophagic cell death, and suppressing the activation of the mTOR signalling pathway[42]. Several therapeutic drugs, such as sulfasalazine[100], thymoquinone[101] and tetrandrine[102] were also shown to induce autophagic cell death and demonstrate an anticancer effect in oral cancer. These results indicated that induction of autophagic cell death is also an alternative approach to killing tumour cells. Therefore, understanding how to overcome cytoprotective autophagy and harness autophagic cell death is critical to enhance the sensitivity of cancer cells to therapeutic agents.

Conclusion and perspectives

Resistance to chemotherapeutic agents is still a major challenge for the success of cancer chemotherapy. Based on the current understanding, a variety of factors including apoptosis, the cell cycle, drug transportation, stemness, DNA damage and repair, EMT and autophagy contribute to innate and acquired resistance in oral cancer. Several new factors also attracted more and more attention in the chemoresistance of oral cancer, such as metabolic reprogramming, tumour microenvironment (tumour-associated macrophages and cancer-associated fibroblast), lncRNAs, circRNA and ceRNA. Accumulating evidence supported the notion that genetics and epigenetic alterations were involved in these processes to develop chemoresistance phenotype. However, the exact molecular mechanisms of chemoresistance are still not fully understood. It is necessary to identify the molecular alterations, elucidate the underlying mechanisms and develop personalised agents for the treatment of oral cancer.

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