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檳榔 genotoxic stress 對 p53 穩定度之影響

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Abstract
Betel squids chewers contribute more than 80% of Taiwan oral cancer patient; the ingredients of betel quids are under studied and the correlation with cancer had became one of the major topic for researchers in Taiwan.

There were several lines of studies for ingredients from betel quids, yet no consistency for the mechanism of oral cancer induced by betel quids. Researchers have been search for the variation on the theme of betel quids-related oral cancers, one of the interesting di fference arises from infrequent mutations in p53 collected from oral cancer patient who are also betel quids chewers. P53 tumor suppressor gene is the most frequent target for genetic alterations in cancer, involves in more than 50% of cancers. The lower mutation incident claimed in betel quids-related oral cancers can be explained, as the mutations in the p53 gene may not play a role in the pathogenesis of Taiwan旧 oral cancer, although this hasn 急 been proved yet. Whereas many tumors that retain wild type p53 show defects in the pathways to stabilize and activate p53, there arise another possibility for the role played by p53 in betel quids-related cancers, which is controlled by posttranscriptional regulation and protein/protein interaction. We found this might be an alternative explanation for the infrequent p53 mutations in Taiwan旧 oral cancer, supported by studies in different system revealing p53 plays a pivotal role in activating and integrating adaptive response to a wide range of environmental stress. Our studies supported by NSC (NSC 89 -2314-B-039-015) demonstrates cells will go through p38 stress-activated protein kinases (SAPK) pathway under betel quids component stimulation, this result explains betel quids stimulation is a genotoxic stress and cells will response to this stress by nature. We then started to consider p53, the universal sensor of genotoxic stress, might be involved in betel quids exercised genotoxic stress. A preliminary experiment showed p53 protein expression was induced after cells were under betel quids component stimulation. According to our preliminary study, we have a hypothesis as following: p53 can response to betel quids induced genotoxic stress, and the accumulation/stabilization of p53 is regulated by

posttranscriptional regulation.

Since posttranscriptional modification of p53 by phosphorylation has been proposed to be an important mechanism by which p53 stabilization are regulated, we will test multisites of phosphorylation by betel quids-specific expression and determine their DNA binding activities in the first year and the second year study. The third year is expected to connect SAPK pathway with p53 activation and particularly find the critical role through phosphorylation.

This study is aimed at finding regulation of p53 stabilization by betel quids in a cellular system. Although the result won 急 be able to answer the question of why infrequent p53 mutations in Taiwan旧 oral cancer patient, yet the result might offer an alternative explanation and understanding for p53 expression under betel quids stimulation.

Background

1. Epidemiology study show betel-quid is associated with oral cancer
Squamous cell carcinoma (SCC) is the most common malignant tumor in the oral cavity and is listed as the fifth prevalent caner. In Taiwan, more than 80% of patient with oral squamous cell carcinoma has betel quids chewing history [1], therefore, ingredients of betel quids were investigated and the correlation with cancer had became the major topic for researchers.

2. Betel-quid ingredients show tumorgenicity
Betel quid is composed of betel nuts (Areca catechu L), mixture with lime and occasionally, tobacco leaf. The different additives are according to regional and individual preference. The International Agency for Research on Cancer concluded that oral squamous cell carcinoma is associated with betel quid chewing together with tobacco or cigarette smoking [10]. In the earlier studies, the generation of areca nut-related N-nitrosamines were suggested playing only a minor role in he etiology of oral cancer among betel quid chewers [11], and controversies existed for the role of tobacoo participating in cancer formation. Whereas tobacco is not included in the betel quids preparation in Taiwan, epidemiological studies show betel quid chewing is still the main cause of oral cancer

in Taiwan, [12] more and more evidences support the notion that betel quids is involved in carcinogenesis of oral cancer.

Among betel-quid carcinogenic investigation, Sundqvist et. al [13] and others [14] show areca nut-related compounds have cytotoxic and genotoxic effects in cultured human buccal epithelial cells. Similar results are also obtained, Jeng et. al [15] showed genotoxic and non-genotoxic effects of betel-quid ingredients on human oral mucosal fibroblasts. When the alkaloids are compared on a weight basis to the extract, no single agent has detectable effects on the cells at concentrations of the extract that cause decrease colony survival and DNA single strand breaks. Therefore, additive or synergistic effects could be considered among the alkaloid [15]. Unscheduled DNA synthesis is suggested by areca nut, inflorescence piper betle extracts and arecoline tin betel quids hewing-related oral mucosal lesions, possibly through both genotoxic and on-genotoxic mechanisms [17].

Besides alkaloid, other classes of components including polyphenols are likely to contribute to the marked toxicity of the extract. Jeng et. al [18] show eugenol, a major polyphenol of betel-quid, is cytotoxic to human buccal mucosal fibroblsts by decreasing cellular ATP level and lipid peroxidation. Safrole is also a major component extracted from betel quids preparation in Taiwan and had been extendedly studied showing DNA adducts formation in vitro by 32P-postlabeling assay, regarded as a genotoxic carcinogen in the rat liver [20]. A recent report further suggests a role of safrole related to oral carcinogenesis, by demostrating safrole forms safrole-DNA adducts in humans oral tissue following betel quids chewing [21].

Tumorgenicity study reveals the aqueous extract of betel nuts is tumorgenic in mice by gavage or subcutaneous injection [22]. Further studies demonstrate arecoline and arecaidine, which are major alkaloid present in betel nuts, are mutagenic in four Salmonella tester strains. Research data from Swiss mice show betel nuts can induce lung tumor. Analyses of betel nuts component show alkaloid and arecoline can form at least four N-nitrosamines under mild nitrosation condition [23]. Some of these metabolites are present in the saliva of betel-quid chewers and found to be potent inducer of benign and malignant tumors of the esophagus, nasal cavity, and tongue in F344 rats.

From the evidences shown above, there is no doubt about the carcinogenticity of betel quids and the carcinogenesis process induced by betel quids ingredient, although the mechanism is still obscured.

3. p53 and oral cancers

Betel quids-related oral cancer has genetic abnormalities including chromosomal aberration and gene dysfunction. Mutations occurred in several genes were investigated. One of the famous gene, p53, has been studied by various groups and has various results.

The p53 gene has been shown to play a major role in wide varity of cellular process, including cell cycle, DNA repair, genome stability, apoptosis, differentiation, senescence. General speaking, the p53 tumor suppressor gene encoded protein plays an important role in the cellular response to stress, and loss of p53 activity is associated with tumor devel opment [46]. Lost or to contain mutations in the p53 tumor suppressor gene occur in about 50% of all human tumors, making it the most frequent target for genetic alterations in cancer[46][47], although there do exist cancers never show p53 mutation [48]. The genetic changes occurred in tumor is usually a missense mutation in one allele, producing a faulty protein that can be observed at high expression, followed by a reduction to homozygosity. About 90% of the mutations in cancers are missense mutations that change the amino acid sequence, resulting in an aberrant protein[49]. About 8% of the mutations are deletions or insertions, 5.5% are nonsense mutations and only 0.8% produces no amino acid changes[50] The mutation sites are best known between exon 5 and 8[51].

In the field of oral cancer research, p53 mutations were also examined and mutations of p53 have been implicated in the pathogenesis of head and neck SCC at a high incidence. Samples from oral SCC patients in Sri Lanka were examined by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and direct sequencing, it was shown mutations in the p53 gene were frequent (10/23) and clustered significantly in exon 5 (7/10) of the p53 gene[52]. Inconsistent result from Taiwan showed infrequent p53 mutations in 2 of 37 (5.4%) patients with areca quid chewing-associated oral SCC and authors suggest that mutations in the p53 gene may not play a role in the pathogenesis of human oral SCCs in

Taiwan[53]. A group from India also showed a lower incidence of p53 mutation was 21% (15 of 72) excluding the polymorphism and the silent mutation [54]. Although the reports states discrepancy of p53 mutation incidence, it is still hard to jump to the conclusion making the consumption of betel quids in these particular areas as the origin of the incidence differences.
The prevalance of p53 expression determined by immunohistochemistry shows various results. The positivity ranged from 11 to 69% in oral SCC[54][55] . Increased p53 protein expression has also brought attention in precancerous lesions of patient chew betel quids and smokers[56]. Detecting p53 protein expression using immunohistochemistry easily arouse questions concerning mutations. Since some p53 antibody recognize both wild type and mutant proteins, it will be impossible to separate the nature between p53 over expression and mutations. Besides, mutations result in deletion or truncation of the protein do not cause protein accumulation, therefore the result will be negative. The immunohistochemistry approach could lead to underestimation of p53 mutations. Regardless of the aforementioned results that support p53 as a cancer marker, most studies haven 急 shown relationship between p53 and clinical parameters.

4. Regulation of p53 stability
There is an alternative mechanism of p53 inactivation besides mutations. The mechanism is in lieu of protein-protein interaction. The p53 is inactivated by abrogating specific DNA binding and/or transactivation activity, sequestering wild-type p53 in the cytoplasm or increasing its degradation[57]. Since p53 activation is a central event in response to different types of stress, it is clear that the triggering of p53 stabilization and activation must be tightly regulated to ensure normal cell function.

Stress from genomic damage can active the constant low concentration of p53, by way of proteolysis mediated by ubiquitin-proteasome system [58]. The accumulated p53 protein occurs mainly through post-translational mechanism without a need for de novo transcription[59]. Rapid post-translational activation is often achieved by covalent modifications, particular protein phosphorylation[60], and the outcome of phosphorylation is the stabilization of p53 through inhibition of p53

ubiquitination and degradation. It is well established that a oncogene, mdm2, plays a major role in p53 stability[61] and p53 mutations do not determine its stability[62]. Further evidence demonstrates mdm2 function as a ubiquitin ligase for p53[63]. The stability of p53 is thought to potentiate genomic stability and consequently inhibit tumorigenesis by cell cycle arrest and apoptosis[46].

5. Other genes related to oral cancer

The expression of mdm2 protein in betel and tobacco related ral malignancies in Indian population, its relationship to linicopathological parameters and p53 protein expression was investigated. The result indicated alterations in dm2 and p53 expression are early events likely to be involved in preinvasive tages in oral tumorigenesis and may be indicative of a 'gain of function' henotype with more aggressive characteristics [28]. Alterations in the p16/pRb pathway [32] and p21ras [39] are investigated and implicated as early events in oral tumorigenesis and may be involved in the development of etel- and tobacco-related oral malignancies. Regarding prognosis, cyclin D1 [30], p53 [31][36], CD44v7-8 expression [33], and Co-overexpression of p53 and c-myc proteins [35] were suggested as prognosis makers of SCC. Besides p21ras overexpressed in SCC and premalignant condition [39], evidence also reveled there is a five- to tenfold increase in amplification of c-myc, N-myc, and Ki-ras. Mutations of Ki-ras oncogene codon 12 were found in 18% of betel-quid chewing-related oral SCC in Taiwan [26]. Amplification of at least one of the oncogenes is found in 56% oral cancer. These oncogenes are known to be associated with other kinases, therefore these oncogenic alteration might influence the downsteam signal transduction.

6. Genotoxic-related signal transduction pathways

Cell respond to extracellular signals by transmitting intracellular instructions to coordinate appropriate responses. One third of mammalian proteins contain convalently bound phosphate and protein kinases represent the largest family of enzyme in humane genome. The important issue about this is nearly all aspects of life are controlled by reversible phosphorylation of proteins, therefore, protein kinases have been implicated in many physiological processes, including cell growth, differentiation, oncogenic transformation, immune responses, and

apoptosis [42][43]. Converge of these kinases can attributed to several kinase pathway, one important and well- studied major kinase pathways is mitogen-activated protein (MAP) kinase pathway. Several MAP kinase pathways have been described in mammalian cells, including the extracellular signal-regulated kinases (ERKs), the Jun N-terminal kinases (JNKs), and the p38 MAP kinases (also termed stress activated protein kinase 2).

Exposure of cells to genotoxic agents evokes a series of phosphorylation events leading to the modification of transcription factors and gene expression. UV irradiation has been the most widely studied genotoxic stimulant, other agents, including the DNA alkylating agent methyl methanesulfonate, result in similar pattern of gene expression. The classical ERK kinase pathway involves activation of cell membrane-associated tyrosine kinases followed by the sequential activation of Ras and Raf. Raf phosphorylates p42/p44MAP kinase kinase (MEK), which in turn activates ERK. The ERKs are members of serine/threonine kinases that are responsible for the phosphorylation and activation of various transcription factors [40]. The JNK pathway relies on the JNK for gene activation following UV treatment. JNKs can also be activated by a variety of stresses and hence are referred to as stress-activated protein kinases (SAPK) [41]. Once activated, JNK will phosphorylate transcription factors, c-jun and ATF2. Therefore, MAP kinase and JNK activation can be regarded as two major indicators of genotoxic-induced signals. The p38 MAP kinase pathway is activated by proinflammatory cytokines and by environmental stress, the substrate includes ATF2 and other transcription factors. These main pathways work as a skeleton, combine with other kinases and proteins makes a full function system. Unraveling the whole network not only can understand the diversity of regulatory mechanism, but also pathological conditions.

Material & Method

According to our preliminary data suggests betel quids related genotoxic stress could induce p53 accumulation. We investigated

1) whether the p53 expression is transcription and translational-dependent

In order to determine betel quids induced p53 expression is a post-transcriptional events, actinomycin D and cycloheximide will be used

as inhibitors of RNA and protein synthesis in advanced to betel quids stimulation. Western blotting will be used for revealing p53 expression.

2) the protein expression level of p53 according to different time course by western blotting.

3) effect of betel quids on p53 mRNA expression and its stability by northern blotting

Western blot analysis

Cell lysates was subjected to electrophoresis through 10% SDS-PAGE gel, and electrophoretically transferred to Immobilon P membranes. P53 mAb Pab421 antibody was used as primary antibody and HRP-conjugated anti-mouse antibody as secondary antibody. Development using the enchanced chemiluminescence system. The amounts was quantified by laser densitometry.

Northern blot analysis

Total RNA was extracted by using Tqizol reagent (GIBCO/BRL) according to the manufacturer protocol

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