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Frequent expression of *MAGE1* tumor antigens in bronchial epithelium of smokers without lung cancer

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Abstract. Melanoma antigens (*MAGE*) are frequently expressed in lung cancer and are promising targets of anti-cancer immunotherapy. Our preliminary data suggested that *MAGE* may be expressed during early lung carcinogenesis, raising the possibility of targeting *MAGE* as a lung cancer prevention strategy. The purpose of this study was to investigate *MAGE* activation patterns in the airways of chronic smokers without lung cancer. *MAGE-A1, -A3* and *-B2* gene expression was determined in bronchial brush cells from chronic former smokers without lung cancer by reverse transcription-PCR (RT-PCR). The results were correlated with clinical parameters. The 123 subjects had a median age of 57 years, a median of 40 pack-years smoking history, and had quit smoking for at least one year prior to enrollment. Among the subjects, 31 (25%), 38 (31%), and 46 (37%) had detectable *MAGE-A1, -A3* and *-B2* expression, respectively, in their bronchial brush samples. Expression of *MAGE-A1* and *-B2* positively correlated with pack-years smoking history (P=0.03 and 0.03, respectively). The frequency of expression did not decrease despite a prolonged smoking cessation period. In conclusion, *MAGE-A1, -A3* and *-B2* genes are frequently expressed in the bronchial epithelial cells of chronic smokers without lung cancer, suggesting that chronic exposure to cigarette smoke activates these genes even before the malignant transformation of bronchial cells in susceptible individuals. Once activated, the expression persists despite long-term smoking cessation. These data support the targeting of *MAGE* as a novel lung cancer prevention strategy.

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Introduction

Melanoma antigens (*MAGE*) have recently emerged as promising immunotherapeutic targets for anticancer therapy, as they are recognized by cytotoxic T lymphocytes (CTLs) in conjunction with MHC class I molecules of various haplotypes on the tumor cell surface. Several clinical trials involving gastrointestinal carcinoma, oesophageal carcinoma, pulmonary carcinoma, melanoma and lung cancer have utilized proteins and peptides derived from some of these antigens and have shown encouraging results (1-3, Vansteenkiste J, et al, J Clin Oncol 25: abs. 7554, 2007).

MAGE antigens are a large number of closely-related proteins (4) classified into type 1 (*MAGE-A*, *MAGE-B*, and *MAGE-C*) and type 2 (*MAGE-D*) based on differences in tissue-specific expression patterns and gene structure (5). While type 2 *MAGE* antigens are almost universally expressed, expression of type 1 *MAGE* antigens has not been reported in normal adult somatic tissues apart from the testis (5,6). However, expression of *MAGE 1* antigens has been documented in a broad variety of malignancies (7-13). These observations have resulted in the general consensus that expression of *MAGE 1* antigens is a tumor-specific phenomenon.

However, our previous research has raised a significant possibility that certain *MAGE 1* antigens are also expressed in the normal lung tissue of smokers with non-small cell lung cancer (NSCLC) (8). We therefore hypothesized that the expression of *MAGE-A1, -A3* and *-B2* antigens is a process initiated in bronchial epithelium exposed to tobacco carcinogenic insult even prior to malignant transformation. To test this hypothesis, we carried out the present study to assess the effect of smoking on the expression of *MAGE* genes in chronic former smokers, with the aim of determining the potential of these antigens as targets for immunotherapeutic approaches in lung cancer chemoprevention.

Materials and methods

Study population. Bronchial brush samples before any chemopreventive intervention were obtained from chronic smokers who had a smoking history of at least 20 pack-years and had

Table I. Patient characteristics and *MAGE 1* expression.

MAGE gene expression		n	Age, years median (range)	PPD median (range)	Smoke-years median (range)	Pack-years median (range)	Quit-years median (range)
-A1	-	92	56.41 (38.52-75.47)	1.5 (0.8-4)	28 (10-58.39)	39.05 (19.98-136)	7.25 (1.01-45.98)
	+	31	61.83 (36.33-73.61)	2 (1-4)	30 (15-50)	55.5 (20-136)	8.84 (1.05-29.26)
P-value			0.3170	0.0676	0.2042	0.0357	0.6132
-B2	-	77	54.52 (38.52-73.62)	1.5 (0.8-4)	27 (10-48)	38.6 (19.98-102)	8.61 (1.02-38.18)
	+	46	62.44 (36.33-75.47)	1.5 (1-4)	30 (14-58.39)	45 (20-136)	7.23 (1.01-45.98)
P-value			0.0066	0.4657	0.0368	0. 0537	0.9501
-A3	-	85	56.87 (36.33-74.51)	1.5 (0.8-4)	28 (10-58.39)	38.6 (19.98-136)	8.84 (1.02-38.18)
	+	38	57.21 (40.49-75.47)	1.5 (1-4)	29.45 (15-50)	46 (21.64-125)	6.93 (1.01-45.98)
P-value			0.9935	0.2993	0.6581	0.1718	0.4969
-A1/-A3	-	108	56.74 (36.33-75.47)	1.5 (0.8-4)	28 (10-58.39)	40 (19.98-136)	7.5 (1.01-45.98)
	+	15	58.96 (41.1-73.61)	1.5 (1-3.5)	27 (15-50)	55.5 (24-125)	7.24 (1.27-23.27)
P-value			0.5503	0.5230	0.4639	0.3798	0.9355
-A1/-B2	-	104	56.08 (38.52-75.47)	1.5 (0.8-4)	27.79 (10-58.39)	39.7 (19.98-136)	7.25 (1.01-45.98)
	+	19	65.11 (36.33-73.61)	2 (1-4)	30 (15-50)	60 (20-136)	10.06 (1.05-29.26)
P-value			0.0375	0.0436	0.0940	0.0135	0.3891
-B2/-A3	-	108	56.74 (36.33-74.51)	1.5 (0.8-4)	28 (10-58.39)	40 (19.98-136)	8.43 (1.02-38.18)
	+	15	58.96 (40.49-75.47)	1.5 (1-3.5)	30 (15-50)	55.5 (22.5-125)	6.62 (1.01-45.98)
P-value			0.5149	0.3832	0.3592	0.1486	0.7902
-A1/-B2/-A3	-	114	56.74 (36.33-75.47)	1.5 (0.8-4)	28 (10-58.39)	40 (19.98-136)	7.5 (1.01-45.98)
	+	9	58.96 (41.1-73.61)	2 (1-3.5)	30 (22-50)	60 (27-125)	7.2 (1.27-23.27)
P-value			0.5386	0.1202	0.3887	0.0561	0.7750

PPD, packs/day.

quit smoking for at least 1 year prior to the time of enrollment. The study was reviewed and approved by the Institutional Review Board, and informed consent was obtained from each subject. Smoking history was recorded as packs/day, smoke-years, and pack-years (the product of packs/day and smoke-years). None of the subjects had any history of lung cancer, and all were clinically free of any cancer at the time of enrollment. Bronchial brush samples were collected from carina through bronchoscopy and placed in culture medium without serum. Samples were immediately transported to the laboratory, washed twice with PBS and stored in aliquots at -80˚C until use.

RNA extraction, RT-PCR and the sequencing of PCR products. Bronchial epithelial cells were used for extracting total RNA using Tri-reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Approximately 1 μg of total RNA from each sample was reverse-transcribed using Superscript Reverse Transcriptase (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol to generate cDNA. To test cDNA integrity, the ß-actin gene transcript was analyzed for each sample as cDNA quality controls. A normal lung cDNA library, NSCLC cell lines (H 1944 for *MAGE-A1* and *-A3*, and H 1792 for *MAGE-B1*) expressing a high level of *MAGE* genes, and genomic DNA from each sample were used as controls in each experiment. To avoid amplification of genomic DNA of the *MAGE* genes, we designed PCR primer sets flanking intron(s): MG1 forward (5'-TGTGGGCAG GAGCTGGGCAA-3') and MG1 reverse (5'-GCCGAA GGAACCTGACCCAG-3') for *MAGE-A1*; MG3 forward (5'-AAGCCGGCCCAGGCTCGGT-3') and MG3 reverse (5'-GCTGGGCAATGGAGACCCAC-3') for *MAGE-A3*; and MGB2 forward (5'-CAGCCAGGGGTGAATTCTCAG-3') and MGB2 reverse (5'-TTCTCACGGGCACGGAGCTTA-3') for *MAGE-B2*. PCR conditions for each *MAGE* gene were 95˚C for 15 min; 35 cycles of 94˚C for 30 sec, 62˚C for 1 min and 72˚C for 1 min; and a final extension at 72˚C for 5 min in a volume of 12.5 μl with 0.5 units of Hotstar Taq Polymerase (Qiagen, Chatsworth, CA). RT-PCR products were separated on 2.5% agarose gels, and the results were read as positive or negative; the data were interpreted by two independent observers to eliminate bias. Representative DNA bands in agarose gels were eluted in 50 μl of sterile water using a QIAamp spin column (Qiagen, Valencia, CA) and directly sequenced using an AmpliCycle Sequencing kit (Perkin-Elmer, Foster City, CA) according to the manufacturer's protocol.

Statistical analysis. Statistical analysis was performed using the χ^2 test or Fisher's exact test for associations between genes and between gene status and other categorical demographic factors. The Wilcoxon rank-sum test was used to analyze differences in median age, packs/day, smoke-years, pack-years, and quit-years between groups. All of the tests were two-sided. A P-value <0.05 was considered statistically significant.

Table II. *MAGE 1* coexpression.

	-B2		
-A1	0	1	Total
0	65	27	92
1	12	19	31
Total	77	46	123
χ^2 test, P=0.002			
	-A3		
-A1	0	1	Total
0	69	23	92
1	16	15	31
Total	85	38	123
χ^2 test, P=0.01			
	-A3		
-B2	0	1	Total
0	54	23	77
1	31	15	46
Total	85	38	123
χ^2 test, P=0.75			

Results

Expression of MAGE antigens in the bronchial epithelial cells of chronic smokers. The 123 subjects consisted of 67 males and 56 females with a median age of 57 years (range 36-75) and a median of 40 pack-years (range 20-136) smoking history (Table I). *MAGE-A1* antigen expression was detected in 31 (25.2%) of the 123 specimens; *MAGE-B2* expression in 46 (37.4%) of the samples, and *MAGE-A3* expression in 38 (30.9%) of the samples. As reported previously, expression of alternative spliced forms of the *MAGE* genes was observed. RT-PCR products were directly sequenced and confirmed to be the expected *MAGE* gene transcripts (data not shown). The primers used in the study did not amplify the corresponding genomic DNAs, excluding the possibility of presenting a pseudogene.

Pattern of coexpression of different MAGE antigens. Of the 123 samples studied, 31 (25.2%) showed coexpression of any two and 9 (7.3%) showed coexpression of all three antigens (Table II). Coexpression of *MAGE-A1* and *-B2* was noted in 19 (15.4%) samples, *MAGE-A1* and *-A3* in 15 (12.2%) samples, and *MAGE-A3* and *-B2* in 15 (12.2%) samples. Importantly, we found a statistically significant pattern of coexpression between *MAGE-A1* and *-B2* (P=0.002) and between *MAGE-A1* and *-A3* (P=0.01). Notably, the significance of the association was stronger for lack of coexpression; i.e., when one gene is not expressed, there is a high chance that the other gene is not expressed either. There was no statistical correlation between the expression of *MAGE-A3* and *-B2*.

Correlation of expression of MAGE antigens with clinical parameters. Expression of the *MAGE* genes in the bronchial epithelium was correlated with subject age, gender, and pack-years smoking history (Table III). *A1* expression was correlated with pack-years of smoking (55.5 vs. 39.1; P=0.04) (Fig. 1A), and *B2* expression with age (median 62.4 vs. 54.5 years; P=0.007), years of smoking (30 vs. 27; P=0.04), and pack-years of smoking (45 vs. 38.6; P=0.05) (Fig. 1B) on univariate analysis. *A1* and *B2* coexpression was correlated with pack-years of smoking (median 60 vs. 39.7; P=0.01); with packs smoked/day (median 2 vs. 1.5; P=0.04) and age (median 65 vs. 56 years; P=0.04) on univariate analysis. On logistic regression multivariate analysis, the expression of *A1* was found to be correlated with pack-years of smoking (P=0.01) after adjustment for gender (P=0.06), *B2* with age (P=0.046) after adjustment for pack-years, and coexpression of any two genes with pack-years (P=0.03) after adjustment for gender (P=0.05). Overall, the expression of *A1* and *B2* together or independently was found to be correlated with pack-years.

Table III. *MAGE 1* expression in logistic regression analyses.

MAGE gene	Parameter	Estimate	SE	Wald 95% CI		P-value
-A1	Gender (M vs. F)	0.8703	0.4637	-0.0386	1.7791	0.0605
	Pack-years	0.0191	0.0076	0.0041	0.0340	0.0123
	Quit-years	-0.0086	0.0245	-0.0566	0.0393	0.7243
-B2	Age	0.0568	0.0260	0.0059	0.1077	0.0288
	Gender (M vs. F)	-0.1994	0.4026	-0.9885	0.5897	0.6204
	Pack-years	0.0116	0.0080	-0.0041	0.0272	0.1472
	Quit-years	-0.0228	0.0248	-0.0714	0.0258	0.3578
-A3	Gender (M vs. F)	0.3706	0.4048	-0.4228	1.1641	0.3599
	Pack-years	0.0062	0.0072	-0.0079	0.0203	0.3904
	Quit-years	-0.0223	0.0229	-0.0671	0.0225	0.3286

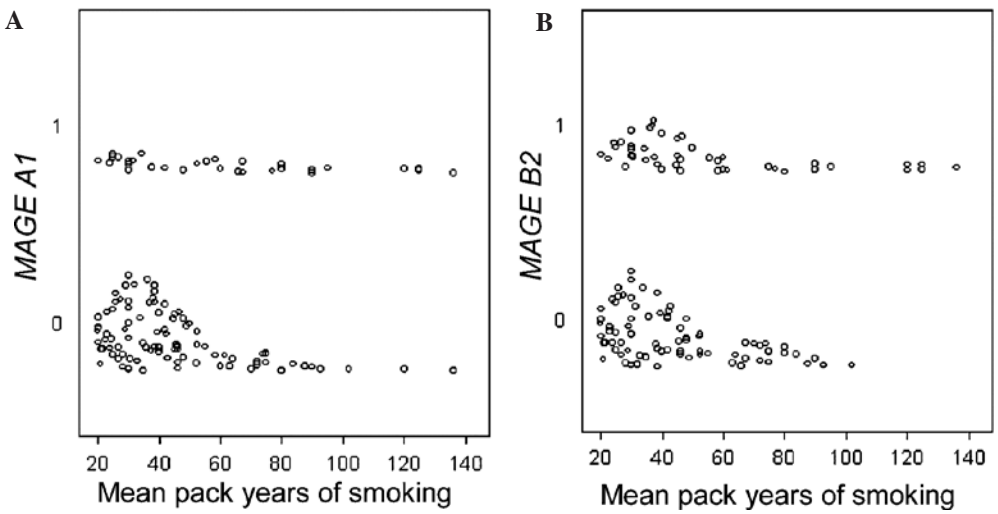

Figure 1. Blip plots of *MAGE-A1* gene expression (1, expressed; 0, not expressed) according to smoking pack-years. Expression is correlated with the mean pack-years of smoking for *MAGE-A1* (55.5 vs. 39.1; P=0.04) and *MAGE-B2* (45 vs. 38.6; P=0.05).

Impact of smoking cessation on the expression of MAGE antigens. To determine the potential impact of smoking cessation on the expression of *MAGE* antigens, the subjects were catgorized into three groups according to the duration of smoking cessation: cessation duration <5 years, 5-10 years, and >10 years. Subsequently, the frequencies of *MAGE* expression among the groups were analyzed using a logistic regression model. Despite a prolonged smoking cessation, no statistically significant decrease in the frequencies of expression of *MAGE-A1* (P=0.6), *-B2* (P=0.8) and *-A3* (P=0.4), respectively, were found.

Discussion

Our results demonstrated that *MAGE-A1, -A3* and *-B2* antigens are expressed in the airway epithelial cells of a proportion of chronic smokers without lung cancer, supporting our hypothesis that these antigens are activated even prior to the malignant transformation of bronchial cells. This novel finding contradicts the current perception that these antigens are expressed only in cancer cells, and has important clinical implications. Since these antigens are not expressed in normal lung tissue, their activation and expression in heavy smokers might represent an early carcinogenic state. Thus, for the first time, this finding draws attention to the novel significance of *MAGE* antigens as chemopreventive targets.

To date, type 1 *MAGE* antigens have been considered tumor-specific (5,6), since they are not expressed in adult normal tissues apart from the testis, (14-16), but are expressed in a wide variety of histologically distinct tumor types (17), including NSCLC (7,8,10). These observations, coupled with the fact that these antigens are immunogenic and can be recognized by autologous cytotoxic T cells (18), have aroused a great deal of interest and suggest these antigens are ideal cancer immunotherapy targets. One clinical trial investigating patients with malignant melanoma demonstrated anti-tumor activity by vaccination with a *MAGE-A3* peptide (1). In a recently completed randomized phase II clinical trial for patients with surgically resected early stage NSCLC, long-term administration of a *MAGE-A3* peptide resulted in an improvement in cancer-free survival (Vansteenkiste J, et al, J Clin Oncol 25: abs. 7554, 2007). This approach is associated with minimal side effects since the testis, the only adult normal tissue expressing *MAGE*, does not express MHC I/II molecules and therefore would be immune-exempt, as germ cells cannot present *MAGE* proteins. Additionally, the testis has a blood-testis barrier, and is therefore considered an immunologically privileged site (13,19). Therefore, immunization of a subject against these antigens, if successful, would be expected to be cell-specific with no direct effects on normal tissues. Thus, immunotherapeutic approaches against these antigens have emerged as a promising and feasible anticancer approach.

However, the present study expands the potential of *MAGE* antigens from a therapeutic to a prevention setting. We analyzed 123 bronchial brush samples from 123 unrelated individual former chronic smokers, which allowed for the determination of the frequencies of *MAGE* antigen activation in this type of cohort, as well as the relationship with smoking history and cessation. Our observations firmly establish that type 1 *MAGE* antigens are expressed in non-malignant adult epithelial cells chronically exposed to tobacco carcinogens. Our findings differ from other reports, which failed to find *MAGE* expression in normal tissues (20,21). This discrepancy is most likely attributable to the use of different primer sets and a smaller amplicon size in our study, which could have affected the PCR amplification efficiency. It is also possible that the normal lung tissues used in other studies may have contained a limited amount of lung epithelial cells, whereas the bronchial brush samples used in our study consisted mainly of lung epithelial cells.

Considering the malignant potential of these antigens, there is a possibility that the smokers who express these antigens are at a higher risk of lung cancer development as compared to other subjects, since these antigens are known to function as oncoproteins. *MAGE-A1* acts as a potent transcriptional repressor by binding to Ski interacting protein and recruiting histone deacetylase 1 (HDAC) (22). Transfection

of a human *MAGE-A3* vector into murine myoblast cells was found to enhance resistance of the cells to apoptosis induced by prolonged ER stress, possibly through its binding and inhibiting murine caspase-12 (23). This suggests that *MAGE-A3* may protect malignant or premalignant cells from apoptosis and, therefore, may provide them with a survival advantage. In fact, *MAGE A3* expression has been found to be associated with lung tumor progression (11).

Expression of *MAGE 1* genes could be indicative of a carcinogenic process, since they have been demonstrated to be linked to overall DNA demethylation (24). Global hypomethylation is a hallmark of most cancer genomes, and is associated with tumor progression (25,26). Thus, it is conceivable that *MAGE-A* expression is linked to progressive disease. This aberrant activation could confer a growth advantage to these cells as compared to normally methylated cells. The acquisition of DNA demethylation by susceptible cells enables them to grow rapidly and to then progressively overwhelm other cells. Targeting these precancerous cells by specific CTL is attractive, since the remaining not-so-transformed cells would probably show less aggressiveness. Thus, a cancer vaccine preventing the emergence of demethylated *MAGE*-expressing precancerous cells could halt the formation and development of cancer.

Although the mechanisms of tobacco-induced expression of *MAGE* antigens are yet to be fully elucidated, it is possible that tobacco carcinogens deregulate the methylation pattern of these genes. Alternatively, they may cause aberrant histone modifications and result in expression of *MAGE* antigens. Bollati *et al* (27) observed that low-dose exposure to airborne benzene was associated with a significant reduction in LINE-1 (-2.33% for a 10-fold increase in airborne benzene levels; P=0.009), AluI methylation (-1.00%; P=0.027) and hypomethylation in *MAGE 1* (-0.49%; P=0.049). In this regard, it is important to note that we recently reported a new subfamily of DNA methyltransferase 3B termed ΔDNMT3B (28). We observed expression of at least seven variants resulting from alternative splicing in bronchial brush samples from cancer-free chronic smokers (data not shown), suggesting that ΔDNMT3B variants potentially regulate DNA methylation in early carcinogenesis. Some of these variants, particularly ΔDNMT3B5, ΔDNMT3B6 and ΔDNMT3B7, which lack the 3' C terminal catalytic domain, may be in competition with other ΔDNMT3B variants, resulting in DNA hypomethylation on pericentromeric satellite regions, thus leading to transcriptional activation of *MAGE* antigens. However, this hypothesis remains to be confirmed. As chronic smokers often present with chronic pulmonary inflammation, such as chronic bronchitis, it is possible that the *MAGE* antigens are activated through the reactive oxygen species and oxidative DNA damage produced by cigarette smoke, which reduces the binding affinity of the methyl-CpG binding protein 2, thereby resulting in epigenetic alterations (29). Although it is possible that certain hematologic stem cells in the inflammation field might express *MAGE* antigens, we previously demonstrated the expression of *MAGE* antigens in non-cancerous bronchial epithelium. Since most of the bronchial brush samples we analyzed contained a high purity of epithelial cells, the expression observed in this study was likely derived from bronchial epithelial cells.

We further assessed the effect of smoking cessation on the expression of these antigens. Notably, the frequencies of the expression were not significantly reduced in those who had quit smoking for more than 10 years compared with those who had quit smoking for less than 5 years, suggesting that the activation of these tumor antigens persists even after long-term smoking cessation. This finding is not surprising, and supplements previous observations that molecular alterations observed in the bronchial epithelium of chronic smokers persist for a long time after the cessation of smoking (30,31). This might be one of the reasons for the fact that former smokers remain at high risk for developing lung cancer (32). Future studies with a quantitative RT-PCR approach will further elucidate the true effect of smoking continuance or cessation on *MAGE 1* expression.

Nevertheless, our findings do suggest that *MAGE 1* antigens are ideal immunotherapeutic targets for lung cancer chemoprevention studies, as they are not expressed in normal lung tissue, but are present in a certain subset of heavy smokers, possibly putting those smokers at a higher risk of lung carcinogenesis than others who lack expression but have a comparable smoking history. Although our study was not designed to determine whether individuals with *MAGE* antigen expression carry a high risk for lung cancer development, the oncogenic properties of these antigens raise the possibility that the expression of *MAGE* antigens may be a biomarker for lung cancer risk. The peptides derived from these antigens may be utilized for immunotherapeutic approaches to lung cancer prevention, and the identification of individual *MAGE* genes would allow for an individualized chemoprevention approach using specific tumor antigens for each subject. However, our results also indicate that the presence of markers is heterogeneous, and a subject expressing one marker may or may not express another. In approximately one-third of the cases, more than one of the subtypes was expressed. The high frequency of coexpression of *MAGE* antigens calls for a polyvalent vaccination approach. Use of multiple immunogens is important, as it increases the probability of inducing a specific immune response and reduces the risk of tumor escape from the immune system by selection of antigen-loss variants.

In conclusion, we demonstrated that *MAGE* antigens are frequently expressed in lungs with chronic tobacco exposure, even prior to overt malignant transformation. Since these antigens have oncogenic properties, smokers who express these antigens might be at higher risk of lung carcinogenesis, and must be considered for a chemoprevention program. The expression of *MAGE* antigens in noncancerous cells indicates that the monitoring of these antigens may be of potential interest for determining new immunotherapeutic agents, and warrants development of widely applicable, polyvalent vaccines. Considering the promising clinical results and the safety profile of *MAGE* antigen vaccines, our findings suggest a possibility of applying the vaccination strategy in a chemoprevention setting for heavy smokers with positive *MAGE* antigen expression.

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