Differential targets of CpG island hypermethylation in primary and metastatic head and neck squamous cell carcinoma (HNSCC)

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Head and neck squamous cell carcinomas (HNSCC) often metastasise to the cervical lymph nodes. It is known for HNSCC as well as other cancers that progression from normal tissue to primary tumour and finally to metastatic tumour is characterised by an accumulation of genetic mutations. DNA methylation, an epigenetic modification, can result in loss of gene function in cancer, similar to genetic mutations such as deletions and point mutations. We have investigated the DNA methylation phenotypes of both primary HNSCC and metastatic tumours from 13 patients using restriction landmark genomic scanning (RLGS). With this technique, we were able to assess the methylation status of an average of nearly 1300 CpG islands for each tumour. We observed that the number of CpG islands hypermethylated in metastatic tumours is significantly greater than what is found in the primary tumours overall, but not in every patient. Interestingly, the data also clearly show that many loci methylated in a patient’s primary tumours are no longer methylated in the metastatic tumour of the same patient. Thus, even though metastatic HNSCC methylate a greater proportion of CpG islands than do the primary tumours, they do so at different subsets of loci. These data show an unanticipated variability in the methylation state of loci in primary and metastatic HNSCC within the same patient. We discuss two possible explanations for how different epigenetic events might arise between the primary tumour and the metastatic tumour of a person.
restriction landmark genomic scanning (RLGS) technique to assess the methylation status of an average of 1293 NotI sites in normal adjacent tissue, primary tumour, and cervical lymph node metastasis from all 13 patients. Although quantitatively we detect a higher proportion of RLGS fragments methylated in the metastatic tumours, this is not simply an accumulation of methylation events since many events found in the primary tumour are not found in the metastatic tumour of the same patient.

MATERIALS AND METHODS

Tissue collection

Primary head and neck squamous cell carcinomas of the oral cavity, larynx, and pharynx, and metastases to the cervical lymph nodes were obtained from the Cooperative Human Tissue Network. All patients were operated on at The Ohio State University and included 11 males and two females ranging from 42 to 77 years of age. The 13 patients’ sample sets were chosen only where the metastatic lymph node contained grossly visible tumour. All normal specimens were harvested from morphologically normal appearing tissue located at least 3 cm from the tumour margin and were used as the normal control tissue for comparisons with the tumour tissues. Pathological evaluation of the normal, primary tumour, and metastatic tissues was performed. All tissues were snap frozen in liquid nitrogen and stored at –80°C before DNA isolation. All tissues were collected under a protocol approved by the Institutional Review Board of The Ohio State University.

RLGS

RLGS gels were run as previously described. For each patient set, the normal profile was compared to the primary tumour profile and to the metastatic tumour profile. In each comparison, RLGS fragments present in the normal profile and absent or greatly reduced in intensity (visual inspection) in one or both tumour profiles from the same patient were scored as methylation events. We have previously determined by comparing quantitative Southern blot analysis with RLGS profile analysis that we can reliably detect loss of spot intensity equal to 30-40% methylation. Such loss events were scored equally as methylation events regardless of whether the loss appeared to be closer to 40% or 100%. Therefore, despite the presence of normal contaminating tissue we are still able to detect methylation in the malignant cells as long as the presence of normal contaminating tissue we are still able to detect methylation in the malignant cells as long as the majority of the tissue used for DNA isolation is malignant. Thus, methylation of a locus in 80% of malignant cells within a tissue with 30% normal cells is well within our detection capabilities. In addition, we and others have previously shown that RLGS spot loss equates with methylation. RLGS fragments were identified by a coordinate naming system previously described.

Polymeric fragments not found in the RLGS master profile used for naming are uniquely named by their section, followed by “XHN” and an arbitrary number (for example, 2DXHN47 indicating section 2D and fragment XHN47).

RLGS fragment cloning

RLGS fragments were cloned using the NotI/EcoRV boundary library mixing gels as previously described. Single pass sequencing was performed from the NotI end of the clone and used for database searches at NCBI. Cpg island characteristics were determined using a CpG island prediction tool at the WebGene web site (http://www-itba.mi.cnr.it/webgene/). All sequencing was performed at the Genotyping and Sequencing Unit (GSU) at The Ohio State University, Division of Human Cancer Genetics.

Bisulphite treatment, PCR, and sequencing

Bisulphite treatment of DNA and subsequent PCR was performed as described by Herman et al with the exceptions that the DNA purification before and after alkali desulphonation was done using the Qiagen gel extraction kit (Qiagen). Bisulphite treated DNA was used as template in PCR reactions set up as previously described. The PCR reactions were run for 35 cycles. The primers used to amplify the promoter region of the formin 2 like gene are forward 5’...TTGGAGTTTGTGAGTAGGG...3’ and reverse 5’...CCCATAAAAAACACAAAA...3’ with an annealing temperature of 59°C. The bisulphite converted sequence of RLGS fragment 2C54 was amplified using the primers forward 5’...GAGGGAGAGGAGAGGAGAGGAGGAG...3’ and reverse 5’...CCACCTCTAATCAAATCATT...3’ with an annealing temperature of 48°C. Methylation sensitive PCR (MS-PCR) for p16 was performed as previously described using the p16M2 and p16U2 primers. Bisulphite sequencing primers for p16 were designed just flanking these MS-PCR primers: forward 5’...GGAGGGAGTTTGGAGTTATTAT...3’, and reverse 5’...CAAACCTCTTACACCCTAAA...3’ with an annealing temperature of 61°C. The PCR products were gel purified using the Qiagen gel extraction kit and cloned using the TA Cloning kit (Invitrogen). Individual clones were sequenced using the M13F primer.

RT-PCR and sequencing

Total RNA was extracted from tissue using a modification of our previously described method. Briefly, specimens were placed in individual plastic bags partly immersed in liquid nitrogen. The bags were resistant to cracking under low temperature exposure. Frozen tissue was crushed by repetitive strikes with a hammer and dissolved in TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) by direct addition to the bag. Specimens were then transferred to centrifuge tubes, followed by freeze-thawing, and debris removed by brief centrifugation. RNA was then extracted according to the manufacturer’s protocol; 1.0 µg of total RNA was used for first strand cDNA synthesis in a total volume of 25 µl and reactions otherwise performed according to the manufacturer’s instructions (ProSTAR, Stratagene, La Jolla, CA). PCR amplification was performed using the Advantage-GC PCR kit (CLONTECH, Palo Alto, CA). Separate reactions were performed for each primer pair with the following reaction conditions: 96°C for one minute followed by 94°C for 30 seconds, 65°C for 30 seconds, 72°C for one minute for 35 cycles (p16), or 33 cycles (p14) and a final five minute extension at 72°C. PCR samples were then run through 2% agarose gels and the presence of amplified product and correct product size verified by ethidium bromide fluorescence in the presence of 100 bp size markers (Gibco BRL, Gaithersburg, MD). Primers were designed by computer analysis (Oligo 4.0, NBL, Hamel, MN) of available DNA sequence for each gene and are intron spanning, precluding PCR amplification of any residual DNA present in RNA samples. Optimum cycle number for PCR amplification was predetermined for each primer set using a mixture of RT reactions from 12 random tumour samples (data not shown). This step is necessary to ensure that PCR amplification remains in the linear range and that production of PCR product does not plateau. Primers used in PCR reactions comprise p16: p16-15, 5’-GGAGGGGGGAGAGGAGGCACG-3’ and p16-14, 5’-TGGCCCTG TAGGACCTTCGGTGAC-3’ and p16-14, 5’-TGGCCCTG TAGGACCTTCGGTGAC-3’ and p16-14, 5’-TGGCCCTG TAGGACCTTCGGTGAC-3’. RESULTS Quantification of hypermethylation phenotypes in primary and metastatic HNSCCs

RLGS Quantification of hypermethylation phenotypes in primary and metastatic HNSCCs

RLGS profiles were prepared from the DNA of primary tumour tissue, normal adjacent tissue, and cervical lymph node metastatic tissue from 13 HNSCC patients. For each patient, the normal tissue profile was compared to both the primary tumour profile and the metastatic tumour profile and RLGS

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fragments were scored for presence or absence in the tumour profiles (see Materials and methods). A total of 33,617 RLGS fragments were analysed in the set of 26 tumours for a mean of 1293/tumour profile with a range of 747-2126. Fig 1 shows portions of RLGS profiles representative of this data set. Note that although the overall intensity varies from gel to gel, spot loss is determined by loss of intensity relative to the surrounding spots. In addition, the fact that spot loss is not 100% in most cases may be explained by partial methylation and/or normal contaminating tissue that is not methylated. In all cases, the non-cancer related normal cerebellum profile shows the normal intensity of the spots, while in some instances the normal adjacent tissue shows slightly reduced intensity (fig 1A, C). Methylation in both the primary tumour and the metastatic tumour was often observed (fig 1A), as was methylation in the metastatic tumour only (fig 1B). More surprisingly, there were many instances where methylation was detected in a patient’s primary tumour, but not in the metastatic tumour from the same patient. Three such examples are shown in fig 1C, D, and E.

We found that 104 RLGS fragments present in at least one patient’s normal tissue profile were absent from either one or
both of that patient’s tumour profiles (table 1). These RLGS fragment losses are indicative of methylation of those loci.23

Within each patient set, we have identified loci that are methylated in the primary tumour only, the metastatic tumour only, or in both (table 1, fig 1). The percentage of RLGS fragment methylation ranges from 0.09% to 2.10% in primary tumours and from 0.36% to 3.30% in metastatic tumours. The frequencies of locus methylation are similar in primary tumours and metastatic tumours; 23 of the 67 methylated in primary tumours (34%) are methylated in more than 30% of the patients and 34 of the 89 loci methylated in metastatic tumours (38%) are methylated in more than 30% of the patients. The differences in the frequency distributions of locus methylation are not statistically significant (Komorogorov-Smirnov goodness of fit test, p=0.23).

The degree of hypermethylation in primary and metastatic tumours is compared in table 1. The combined data from all 13 patients indicate that 0.99% of the RLGS fragments are methylated in the primary tumours, while 1.47% are methylated in metastatic lymph nodes. A total of 167 methylation events out of 16 868 data points were detected in primary tumours, and 246 methylation events out of 16 749 data points were detected in metastatic tumours. An unpaired Z test using the normal approximation to the binomial distribution to compare the frequencies of methylation between primary and metastatic tumours indicates significantly more methylation in the metastatic tumours as a group (p<0.001).

Table 1  Patient and RLGS analysis data for 13 HNSCC patients

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<th>Patient</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Race</th>
<th>Tissue</th>
<th>% of analyed loci methylated</th>
<th>% of analyed loci methylated</th>
<th>% of analyed loci methylated</th>
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<td></td>
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<td>Metastatic</td>
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<td>+Metastatic only</td>
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| *Number of loci methylated in the primary tumour, but not the metastatic tumour. |
| †Number of loci methylated in the metastatic tumour, but not the primary tumour. |
| ‡Number of loci methylated in both the primary and metastatic tumours. |
| §Number of loci that were methylated in one tumour, but unable to be analysed in the other tumour RLGS profile. |
| ¶Number of loci methylated in the primary only divided by the number of loci methylated in primary only plus the number methylated in both primary and metastasis. |

Primary and metastatic tumours show methylation of different loci

Additional information can be gleaned by studying the distribution of locus hypermethylation in each individual patient set (table 1). With the exceptions of patients 2 and 29, the degree of overlap between loci methylated in the primary tumour and the metastatic tumour is surprisingly small. Patients 4, 7, 8, and 32 exhibit more than 10 loci that are methylated in their primary tumours, but are no longer methylated in their metastatic tumours. These observations cannot be explained by contaminating normal tissue masking our ability to detect methylation since numerous other loci are methylated in these metastatic tumours. The percentage of loci methylated in the primary tumour of a patient, but not in the metastatic tumour from the same patient range from 17% to 93% (table 1). These data indicate that the group of loci that become hypermethylated in HNSCC tumours may differ significantly between the primary tumour and metastatic tumour of an individual person and, furthermore, that this difference is highly patient specific.

One hundred and four methylated loci have been identified in this patient set. By combining the data from all 13 patient sets, we are able to look for trends in the methylation state of specific loci. Eight loci are methylated exclusively in primary tumours, 17 in metastatic tumours, and 79 in both the primary and metastatic tumours; 88 of the 104 methylated loci in this data set are lost in more than one tumour. The distribution of the methylation events found in the 26 tumour samples is shown in fig 2 for these 88 loci. There are 86 instances where a locus is methylated only in a patient’s primary tumour, 170 only in a patient’s metastatic tumour, and 73 in both the primary and metastatic tumours of an individual patient.

Cloning and characterisation of targets of hypermethylation

Fifteen hypermethylated loci were readily clonable using the NotI/EcoRV boundary library and mixing gel techniques previously described.6 All 15 were confirmed by mixing gel analysis. Chromosomal location, CpG island traits, and the genomic context of these 15 loci are shown in table 2. All 15 loci fall within CpG islands. Homologies to known genes were identified for six loci, to ESTs for six, and to a pseudogene for one locus. For all six known genes, the CpG island from which the RLGS fragment arises is found in the promoter region and 5’ end of the gene.

Methylation differences between primary and metastatic tumours are confirmed by bisulphite sequencing

There were 93 instances where an RLGS fragment was methylated in the primary tumour, but was not methylated in the metastatic tumour from the same patient (table 1, fig 1C, D, E). To investigate this surprising finding in more detail, an RLGS fragment (2C54) and a tumour suppressor gene previously identified as a target of hypermethylation in HNSCC (p16) were further analysed. RLGS profile analysis identified fragment 2C54 as methylated in the primary tumour of patient 23 but not the metastatic tumour.
Bisulphite sequencing primers were designed to amplify a region within the 2C54 CpG island containing 49 CpG dinucleotides. Fifteen normal, 17 primary tumour, and 24 metastatic tumour clones were sequenced from patient 23. More than twice as much methylation was detected in the primary tumour clones as compared to the metastatic tumour clones. The proportion of clones showing methylation was calculated for each CpG dinucleotide and is plotted in fig 3. The proportion of methylated clones is greatest in the primary tumour throughout the entire set of 49 CpGs studied. The level of methylation detected in the normal tissue and the metastatic tissue overlaps considerably. It is important to note that these data represent a relative quantification, not an absolute quantification. Owing to the previously reported possibility for PCR bias in bisulphite sequencing, we cannot use this method for absolute quantification. The low absolute level of methylation detected here is likely to be a reflection of the relative difficulty of amplifying the methylated product, since it contains up to 49 CpGs within 332 bp.

**p16 study provides independent confirmation of methylation unique to a patient’s primary tumour**

The previous data have shown the phenomenon of methylation present in the primary tumour, but no longer present in the metastatic tumour of the same patient at anonymous RLGS loci. In order to understand better the biological significance of this phenomenon, we looked to see if we could find it in a well characterised tumour suppressor gene whose loss of function has been clearly shown to have biological significance. The *p16* gene promoter has been previously identified as

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**Figure 2** Distribution of methylated loci by patient. Black indicates methylation. White indicates lack of methylation. NA indicates that the fragment was not analysable. P indicates primary tumour. M indicates metastatic tumour. PO indicates the number of patients where loss was in the primary tumour only. MO indicates metastatic tumour only. Both indicates where loss was in both.
a target of CpG island hypermethylation in HNSCC and this methylation has been correlated with silencing of the gene. Methylation specific PCR (MS-PCR) was performed for the p16 promoter as previously described\textsuperscript{32} in a set of nine primary and metastatic tumours. Of the nine patients studied in fig 4A, only patient 4’s primary tumour showed methylation. No methylation is detected in the metastatic tumour from patient 4; however, the presence of the unmethylated p16 band indicates that the locus has not been homozygously deleted. The MS-PCR result was confirmed by bisulphite sequencing of 10 clones each for normal adjacent, primary tumour, and metastatic tumour DNA from patient 4 using primers designed to flank the region amplified by MS-PCR. The primary tumour DNA showed heavy methylation of approximately half the clones, while the metastatic tumour showed almost no methylation (fig 4B). Methylation of multiple other loci, as detected by RLGS analysis of the metastatic tumour from patient 4, shows that the ability to detect methylation in this tumour is not masked by contaminating normal tissue.

RT-PCR from RNA obtained from the primary and metastatic tumours from patient 4 showed low expression from the primary tumour where methylation was detected, but high expression from the metastatic tumour where no methylation was detected (fig 4C). The primers used for the p16 RT-PCR reaction fall in exon 1 of the p16 transcript and exon 2 shared by both the p16 and p14\textsuperscript{ARF} (p14) transcript. Direct sequencing of the RT-PCR product (the entire p16 transcript) showed that the transcript expressed in the metastatic tumour has a 17 bp deletion early in exon 2 (nucleotide 219 of the p16 mRNA), resulting in a frameshift (fig 4D). The frameshift occurs after amino acid

<table>
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<th>RLGS fragment</th>
<th>Chr location</th>
<th>Size (bp)</th>
<th>%GC</th>
<th>CpG:GpC</th>
<th>Genomic context</th>
<th>Class of methylation</th>
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Table 2  RLGS fragment sequence characteristics

![Figure 3](Figure 3  Bisulphite sequencing of RLGS fragment 2C54. After bisulphite PCR of 2C54 from patient 23’s HNSCC DNA, 15 normal adjacent tissue clones, 17 primary tumour clones, and 24 metastatic lymph node clones were sequenced. The data are presented as the proportion of CpGs methylated per group of clones for each of 49 CpG dinucleotides sequenced from the 332 bp cloned products. The proportions were calculated by dividing the number of methylated CpGs by the number of clones sequenced from that tissue. The proportion of methylated CpGs is represented on the Y axis, and each of 49 CpGs is represented on the X axis.)
and produces a nonsense mutation with a stop codon at amino acid 112. The low level transcript detected in the primary tumour has no mutation.

The transcript detected in the primary tumour may arise from contaminating normal cells, and so to determine if the 17 bp deletion is present in the primary tumour cells the transcript was studied. RT-PCR was performed on the same samples using a primer specific to the exon 1, and the same second primer in the shared exon 2 that was used for RT-PCR. The transcript is equally expressed in both the primary and the metastatic tumours of patient 4 (fig 4C). Furthermore, direct sequencing of the RT-PCR product shows that, just as with the transcript, the metastatic tumour has a 17 bp deletion while the primary tumour transcript has no mutation. These results confirm that in patient 4's primary tumour there is methylation of the p16 promoter, but no mutation, whereas in the metastatic tumour from the same patient, there is mutation of p16 but no methylation. Important to the biology of the tumours, however, both genomic alterations lead to loss of function of the p16 protein.

**DISCUSSION**

A crucial point in our understanding of cancer biology is that neoplastic cells evolve from the accumulation of genetic changes that give the cells a growth or survival advantage. These genetic changes tend to accumulate in a loose order. For instance, loss of chromosome 9p is a common and early change (often in hyperplasia) in the development of HNSCC, while loss of chromosome 4q is found late (often in CIS and invasive tumour) in HNSCC. It seems reasonable to predict that epigenetic changes such as CpG island hypermethylation may also accumulate in a similar manner. To address this idea experimentally, we have used restriction landmark genomic scanning (RLGS) to study the hypermethylation phenotypes in 13 HNSCC patients, comparing the RLGS profiles from their primary tumours and cervical lymph node metastases to their normal adjacent tissue profiles. CpG island hypermethylation is generally thought to result in loss of function of the gene involved owing to suppression of transcription. This is analogous to other loss of function mutations such as deletions and point mutations. Deletions resulting from unbalanced translocations are the most common type of mutation in HNSCC. Nevertheless, it is clear from the data presented in this article, as well as the data of others, that the mutation spectrum in HNSCC also includes CpG island hypermethylation. We found that in the overall data set there is a small but significant increase in the degree of CpG island hypermethylation in metastatic tumours compared to the primary tumours. Importantly, however, we also found that the hypermethylated

![Figure 4](image_url)
loci in primary tumours and metastatic tumours can vary tremendously within a single patient. Thus, even though there tends to be more methylation in any one patient’s metastatic tumour, this is not simply the result of an accumulation of additional methylation events, since many found in the primary tumour are no longer found in the metastatic tumour. The percentage of specific loci that are methylated in the primary tumour of a patient, but not in the metastatic tumour from the same patient varies widely from 17% in patient 29, to 93% in patient 4, with an average of 58% (table 1). This indicates that there is not an accumulation of epigenetic changes in the metastatic tumours, but instead a different set of changes with only a portion of those found in the primary tumour retained in the metastatic tumour. This is reinforced by the bisulphite sequencing data of RLGS fragment 2C34 and the p16 promoter. Furthermore, the finding of very low p16 expression in the methylated primary tumour, but high expression in the unmethylated metastatic tumour, and that the metastatic tumour harbours a genetic mutation in p16 while the primary tumour does not, offers some insight on how to explain these findings.

One interpretation of these data would be to suggest that there is substantial epigenetic heterogeneity within the primary tumours. We would then predict that hypermethylation events present in both the primary and metastatic tumours are from events that were present very early in the development of the primary tumour. Further evolution of the tumour may proceed differently in various regions, either by random chance or by selection because of different microenvironments. This may result in the formation of many unique subpopulations with regard to methylation within the primary tumour. Since our sensitivity with RLGS is limited to reliably detecting 30% hypermethylation of a locus, the hypermethylation events we have detected in primary tumours are likely to be present in most subpopulations of cells within the tumour. If one of the minor subpopulations of cells with differential hypermethylation events acquires characteristics advantageous to metastatic spread, then the hypermethylation we detect in the metastatic tumour would be different from what is detected in the primary tumour. The differential methylation events may or may not directly contribute to the cells’ propensity for metastasis.

The fact that patient 4’s primary and metastatic tumours have found different ways to inactivate p16 function fits well within this interpretation. Perhaps because of a field effect, multiple initiated cells became malignantly transformed and ultimately contributed to the primary tumour. Some of those cells may have inactivated p16 function by promoter hypermethylation and contributed to the majority of the primary tumour, while other cells had a 17 bp deletion and made only a minor contribution to the population of primary tumour cells. However, this minor population continued its independent evolution and acquired the ability to create the metastatic tumour in the cervical lymph node.

An alternative interpretation of the data relies on the potential plasticity of DNA methylation. Although DNA methylation can act as a loss of function mutation or as point mutations or deletions, a significant difference is that while genetic mutations are permanent, hypermethylation of a CpG island may be reversible. An excellent example of how such a scenario may be advantageous to tumour development has been proposed for the E-cadherin gene (CDH1). Hypermethylation in homotypic cell-cell adhesion of differentiated epithelial cells, and loss of expression has been linked to invasiveness. It is advantageous for a cell to express CDH1 when growing in a tumour mass, but in order to become invasive, or to leave the tumour mass for metastatic spread, it is advantageous to block expression. Graff et al12 have shown that the prostate cancer cell line TSP1P1 shows moderate levels of hypermethylation of the CDH1 locus. When this cell line is put into a model culture system for basement membrane invasion, the invasive cells show higher levels of hypermethylation. However, when this cell line is put into a culture model system for three dimensional tumour growth, the cells showed low levels of hypermethylation. This potential for dynamic hypermethylation of promoters affords the opportunity to up- or downregulate gene expression either randomly or in response to other signals. This represents a significant difference between epigenetic and genetic changes.

The lack of hypermethylation seen in the metastatic HNSCC tumours at loci that were previously methylated in the primary tumours may in part be explained by dynamic loss of hypermethylation. This idea, and the idea of the independent development of subpopulations of cells with unique hypermethylation patterns within the primary tumour, is not mutually exclusive. Our findings show that the patterns of CpG island hypermethylation in the primary and metastatic HNSCC are complex and suggest that the relationship between those patterns in any one patient may require a more sophisticated explanation than a simple accumulation of changes.

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