ONLINE MUTATION REPORT

Diagnosis of gene dosage alterations at the *PMP22* gene using MAPH

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J Med Genet 2003;**40**:123 (http://www.jmedgenet.com/cgi/content/full/40/11/123)

Genomic rearrangements due to submicroscopic duplications or deletions are responsible for many inherited disorders, and a gene (or genes) sensitive to dosage alterations may be involved in these structural rearrangements. The segment involved is generally smaller than 2 Mb and cannot be detected by conventional karyotyping.[1] Charcot-Marie-Tooth disease type 1A (CMT1A) [OMIM #118220] and hereditary neuropathy with liability to pressure palsies (HNPP) [OMIM #162500] are well characterised as genomic disorders.[2] By a gene dosage mechanism, CMT1A or HNPP respectively result from duplication or deletion of a 1.4 Mb DNA fragment in 17p12 containing the *PMP22* gene. Amplification of the *PMP22* gene from two copies in normal people to three and even (in severe cases) four copies has been detected in CMT1A cases, and deletion of the gene to one copy results in HNPP. CMT1A/HNPP are caused by a reciprocal unequal crossover event arising from the misalignment of homologous repeat sequences, CMT1A-REPs, which flank the 1.4 Mb region. These 24 kb duplicons are 98.7% identical and are thought to mediate this misalignment.[1 3] In a minority of cases, point mutations in the same gene can also result in CMT1A or HNPP.

These two distinct conditions are autosomal dominant traits in a clinically and genetically heterogeneous group of peripheral neuropathies termed hereditary motor and sensory neuropathies (HMSN). The overall estimated prevalence of HMSN is about 1 in 2500,[4] and CMT1A is the most common inherited peripheral neuropathy, with a prevalence of 10–40/100 000.[5] A European collaborative study reported the duplication in 70.7% of unrelated CMT1A patients and the deletion of the same region in 84% of HNPP cases.[6]

There is a wide range of approaches for molecular diagnosis of CMT1A and HNPP. These approaches can be classified as (*a*) methods detecting rearrangements and (*b*) quantitative methods. In the first group, pulsed field gel electrophoresis (PFGE, the standard approach in the USA) and fluorescence in situ hybridisation (FISH) can detect these duplications and deletions,[7] and PCR methods detect specific junction fragments between CMT1A-REPs. Restriction fragment length polymorphism Southern blot analysis and genotyping of short tandem repeat (STR) markers from the CMT1A/HNPP region are methods belonging to the second group. PFGE depends on high quality DNA from patients' blood samples. This labour intensive approach can cause an estimated failure rate (non-interpretable results) of about 10%.[5] The fact that only about 70% of the CMT1A duplication carriers demonstrate the junction fragment should be considered when using diagnostic methods that rely on detection of a junction fragment. In interphase FISH for CMT1A, a minimum of 50 nuclei should be counted for relative signals and 10–30 nuclei for HNPP in metaphase FISH. A failure rate of 10–30% was estimated in some laboratories for reasons such as a reduction in the quality of the prepared nuclei.[5]

Characteristics of an ideal diagnostic test include technical simplicity, accuracy and reliability—that is, not producing test failure or uninformative results frequently. Routine methods to detect the duplication in CMT1A patients have been compared in UK diagnostic laboratories.[8] The aim of that study was to test the sensitivity and reproducibility of each method. The five approaches compared in the study were: microsatellite analysis; junction fragment detection either by Southern blot or PCR; and STS dosage either with capillary electrophoresis or polyacrylamide gel electrophoresis (PAGE). None of these methods was ideal; methods based on a junction fragment missed 14% of duplications, microsatellite analysis had a high failure rate and several tests were required, and STS dosage methods often needed repeating. Rowland *et al*[8] concluded that STS dosage analysis was the most sensitive method but is technically more difficult than the other methods. Other gene dosage approaches such as array comparative gene hybridisation or multiplex amplifiable probe hybridisation (MAPH) might preserve the high sensitivity in a more robust assay format.

With the currently available technology, molecular genetic diagnosis still remains a labour intensive and costly procedure. We therefore applied MAPH to DNA based

Key points

- There are different approaches to assay dosage alterations at the *PMP22* gene, leading to CMT1A (duplication) or HNPP (deletion), all of which have limitations.
- We applied multiplex amplifiable probe hybridisation (MAPH) to the *PMP22* gene in order to develop a reliable and sensitive test for detecting these gene dosage changes.
- The method was used in a blind test on 62 samples collected for a previous comparative study on routine methods for CMT1A diagnosis used in UK laboratories. Apart from three minor discrepancies, all diagnoses agreed with results from other methods.
- A further blind test on 10 samples successfully distinguished the HNPP cases from unaffected samples.
- The MAPH *PMP22* assay is a simple, fast and accurate screening test for molecular diagnosis of CMT1A and HNPP.

Abbreviations: CMT1A, Charcot-Marie-Tooth disease type 1A; FISH, fluorescence in situ hybridisation; HMSN, hereditary motor and sensory neuropathies; HNPP, hereditary neuropathy with liability to pressure palsies; MAPH, multiplex amplifiable probe hybridisation; MRD, mean relative dosage; PAGE, polyacrylamide gel electrophoresis; PFGE, pulsed field gel electrophoresis; RFU, relative fluorescent units; STR, short tandem repeat

measurement of copy number at *PMP22*. MAPH is a simple method based on hybridisation and PCR.[9–12] Submicroscopic alterations can be examined by using a set of short probes (140–600 bp) all flanked by binding sites for the same primer pairs. Such probes can be recovered and amplified quantitatively following hybridisation to a genomic target. After stringent washing, specifically bound fragments are amplified using the common primers, and the differently sized products resolved and quantified after polyacrylamide gel electrophoresis. As an excess of probe is present, the amount of probe amplified depends on the number of hybridising targets and therefore on the copy number of the corresponding locus in the test DNA.

The advantages of MAPH include high resolution, high multiplicity (up to 60 loci at a time) and high throughput (up to 46 samples per experiment). In this study, we applied MAPH to the *PMP22* gene to develop an efficient and sensitive test for determining DNA dosage in CMT1A/HNPP patients.

MATERIALS AND METHODS

PMP22 probe set

A set of 19 probes was developed for the MAPH *PMP22* assay. The set consists of seven probes from the *PMP22* gene (fig 1), nine probes from other autosomes, two probes from the sex chromosomes and a non-human DNA probe. The nine probes from unlinked autosomal loci (from the *TBX5* gene and subtelomeric regions) acted as a reference framework, and the sex chromosome probes (from the 41 probe set described in[9]) and the non-human probe acted as controls for specificity of hybridisation and washing. *PMP22* probes CMT5, CMT9, and CMT10 were subcloned from a 3′UTR fragment, and probes PMP1A, 2, 3, and 4 from the corresponding exons of *PMP22*. Fragments were amplified from genomic DNA and amplicons cloned into the *Eco*RV site of pZErO-2 (InVitrogen, Paisley, UK). All the *PMP22* probes were verified by sequencing, and conformed to the sequence of GenBank (accession no. AC005703). DNA sequences for all these MAPH probes can be obtained from http://www.nottingham.ac.uk/~pdzjala/maph/maph.html. The final probe mixture was column purified and adjusted to a final concentration of about 1ng/µl of each probe.

Hybridisation conditions

Details of established methodology for the MAPH assay have been published elsewhere,[9 11] and updated details are available from the website above. Briefly, denatured genomic DNA was immobilised on small nylon filters. Denatured probe mixture was added to the filters (10–15 per hybridisation) in 200 µl hybridisation solution and incubated at 65℃ overnight (14–18 hours). After hybridisation, the filters were washed at 65℃ to a final stringency of 0.1× SSC, 0.1% SDS. After washing, filters were transferred to 0.2 ml thin walled tubes for denaturation at 95℃ for 5 minutes; then 1 µl of this primary product was used as input for fluorescent PCR using the flanking primers PZA 5′-AGTAACGGCCGCCAGTGTGCTG-3′ (5′-FAM labelled) and PZB 5′- CGAGCGGCCGCCAGTGTGATG-3′.

Of the PCR product, 1.5 µl was mixed with 1.5 µl ROX labelled G-500 marker in loading dye, and denatured (95℃, 3 minutes); of this, 1.5 µl was loaded on a standard 'long ranger' gel in an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and run for 3 hours.

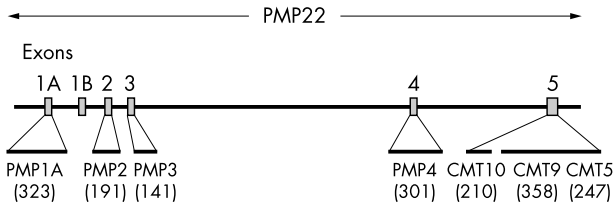

Figure 1 Positions of *PMP22* MAPH probes relative to *PMP22* exons. The numbers in brackets are the probe lengths in base pairs.

Quantitative analysis

GeneScan software was used to record peak areas corresponding to the signal from each probe. In order to produce normalised ratios reflecting the relative probe dosage, each peak area was divided by the sum of the four nearest peaks from the autosomal reference group. For each probe, this ratio was divided by the mean of same ratio from unaffected controls run in the same experiment. Expected normalised ratio (NR) values are 1.0 in the absence of copy number change, and 0.5 and 1.5 in the case of heterozygous deletion and duplication, respectively.

Quality control and diagnostic criteria

According to the ABI GeneScan Reference Guide, best results are obtained if the fluorescent signals range between about 150 and 4000 relative fluorescent units (RFU). Our experiments showed that reproducible results are obtained when all peak heights (except for the Y chromosome and the two longest probes (19p, 18q)) are more than 50 RFU. To screen out samples with extreme PCR bias towards smaller fragments, if the ratio (19p+18q)/(B1+7p)—that is, (peak number 1+2)/(peak number 6+7) in a gel lane was less than 0.3, this trace was excluded from the analysis. To ensure specificity of hybridisation and washing, the signal from non-human DNA probe should have a peak less than 20% of the average of autosomal control probes.

RESULTS

To develop a sensitive and specific assay, we aimed to measure dosage at *PMP22* using the combined results from seven probes. After initial trials, probe CMT9 was excluded from further analysis becausee it gave unreliable results, presumably due to cross-hybridisation to other loci. Thus our measure of *PMP22* dosage was based on the combined relative dosage of six *PMP22* probes. This measure was the mean relative dosage of six different probes from *PMP22*, which we call MRD6.

To test the fundamental specificity and reliability of our probes before more extensive characterisation, MAPH experiments were carried out on genomic DNA of 94 unaffected controls, together with one sample containing a known *PMP22* duplication and one sample containing a deletion associated with HNPP (fig 2). In this test, each DNA sample from unaffected controls was tested in duplicate, and the value reported is the mean of the two MRD6 measurements. As can be seen from fig 3, for unaffected controls the means of the duplicate MRD6 measurements showed an approximately normal distribution, with an observed mean of 0.995 and standard deviation of 0.07. The two positive control DNA samples (CMT1A and HNPP in fig 3) had MRD6 values of 1.43 and 0.56, respectively. These are significantly different from same ratio in 94 unaffected controls ($p<0.001$ in both cases).

Using MRD6, unknown samples could be categorised as: (*a*) normal (between 0.75 and 1.25), (I) deleted (<0.6), and (*c*) duplicated (>1.4). For results in uncertain ranges (0.6–0.75 and 1.25–1.4), the MAPH test could be repeated. If samples are tested in duplicate, and the average of two MRD6 measures has a normal distribution with a mean of 1 and a standard deviation of 0.07, the predicted rate of false positive duplications and deletions is less than 0.001%, and the

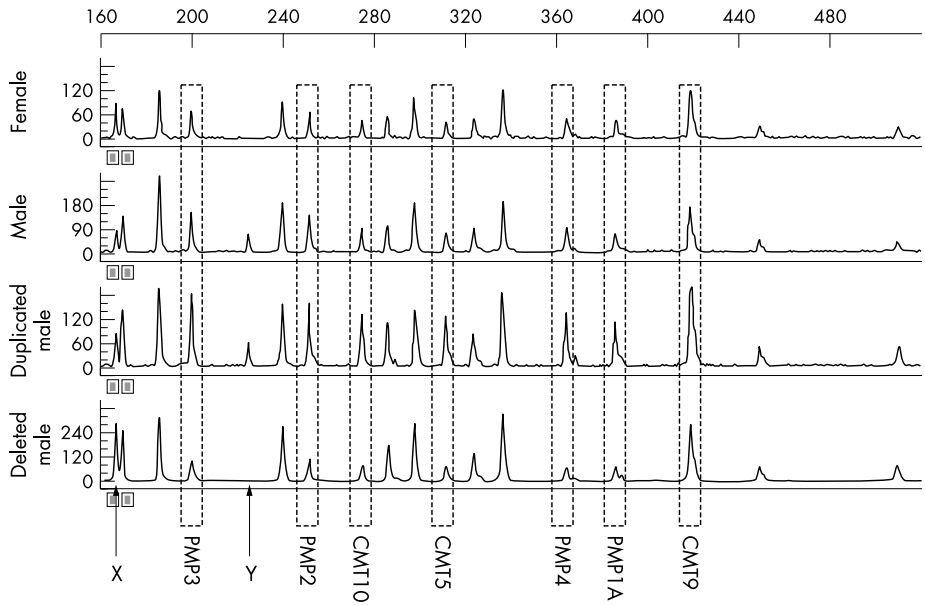

Figure 2 Gel trace from MAPH analysis of DNA from two unaffected controls compared with DNA from a patient with CMT1A (duplicated) or with HNPP (deleted).

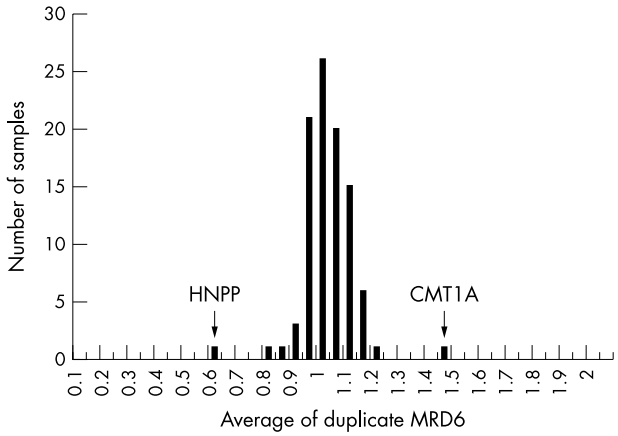

Figure 3 MAPH results from 94 unaffected controls (each tested in duplicate) and two positive controls (CMT1A and HNPP). The MRD6 score used is the mean relative dosage of six probes from *PMP22*, and for each sample the mean of two duplicate MRD6 results is shown. Results from unaffected controls showed an approximately normal distribution, with a mean of 0.995 and standard deviation of 0.07.

Table 1 Detail of MAPH results for sample H1 in four different experiments, showing that there is no single experiment or probe indicating any duplication

	Experiment				
Probe	1	2	3	4	Mean
19p	1.17	0.77	1.22	1.12	1.07
18q	1.10	0.83	1.14	1.00	1.02
CMT9	**1.24**	**0.96**	**0.80**	**0.93**	**0.98**
PMP1A	**1.25**	**1.15**	**0.88**	**1.21**	**1.12**
PMP4	1.31	1.09	1.08	0.96	1.11
B1	0.79	1.14	0.79	0.96	0.92
7p	0.98	0.95	1.04	1.22	1.05
CMT5	**1.26**	**0.97**	**0.93**	**1.16**	**1.08**
Da	1.19	1.05	0.97	0.91	1.03
8p	0.87	1.06	1.01	0.89	0.96
CMT10	**1.06**	**1.41**	**1.16**	**1.41**	**1.26**
PMP2	**0.99**	**1.14**	**1.11**	**1.13**	**1.09**
2q	0.98	1.06	1.06	0.99	1.02
PMP3	**1.08**	**1.29**	**1.19**	**1.22**	**1.20**
19q	0.96	0.72	0.90	0.93	0.88
A2	1.17	1.30	1.30	1.38	1.29

MAPH probes from the *PMP22* gene contributing to the dosage statistic "MRD6" are in **bold**.

predicted incidence of normal samples falling into the uncertain ranges is about 0.04%.

A blind test on 10 samples successfully distinguished five HNPP cases from unaffected samples (data not shown). Another test was set up on a coded set of 73 samples used in the comparative study on routine methods for CMT1A diagnosis.[8] MAPH gave technically satisfactory duplicate results from 62 of the samples (30 normal, 31 duplicated and 1 uncertain (repeat test indicated)), a single result from five (two normal and three duplicated) and no result from six. Failure to produce duplicate results from all 73 samples was due to the limited quantity of the remaining DNA samples. Fig 4A shows the distribution of duplicate mean MRD6 values in these 62 samples. Apart from three discrepancies described below, all diagnoses agreed with results from other methods used by Rowland *et al*.[8] The discrepant samples were identified as H1, H38, and H39, with mean MRD6 values of 1.11, 1.63, and 1.29, respectively. According to our criteria, we declared H1 as normal, H38 as duplicated, and H39 as uncertain. The Rowland *et al* study[8] reported both H1 and H38 as partial duplication, and H39 as normal.

The results of four different MAPH analyses were consistently normal for sample H1 as two duplicate tests were carried out (table 1). Although there are individual *PMP22* dosage measurements ranging from 0.88 to 1.41, these are mostly within the expected normal range for an assay, with a standard deviation of approximately 0.1, and the MRD6 values are entirely compatible with the distribution for unaffected control samples; indeed, they are centrally placed within it. In the Rowland *et al* study, normal results were reported for this sample for microsatellites and by the detection of junction fragments using both Southern blotting and PCR, but duplicated results were obtained with both

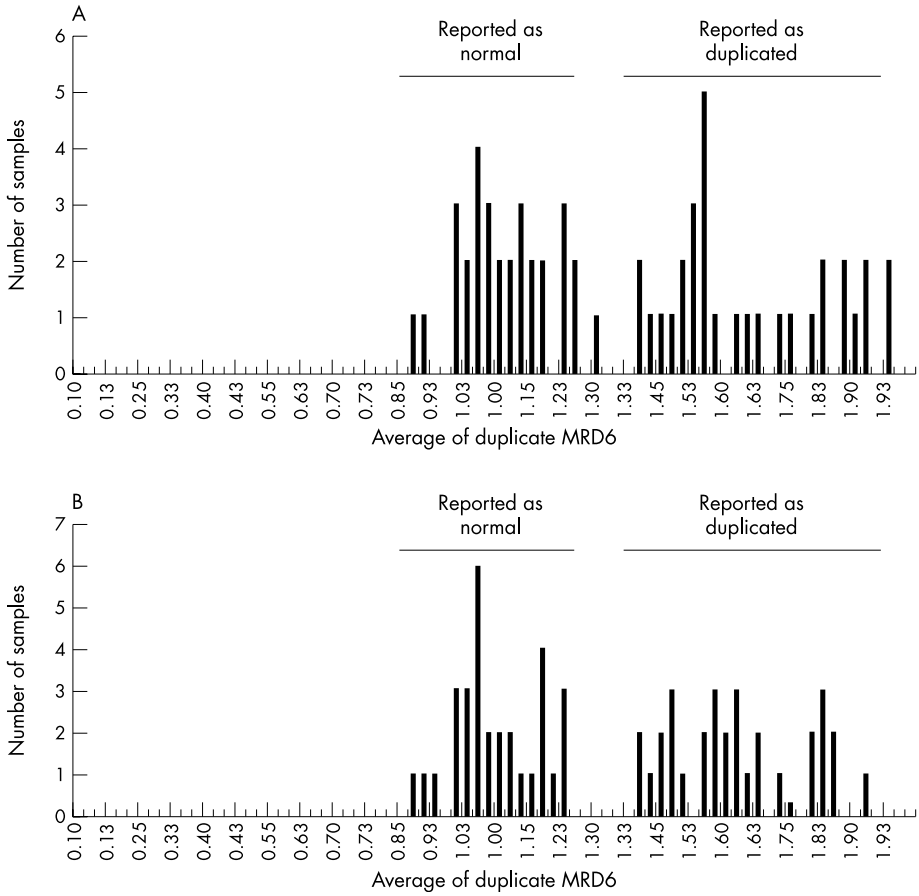

Figure 4 (A) MRD6 analysis of 62 samples in duplicate, normalizing to four neighbouring autosomal probes as reference framework. (B) Alternative analysis of MRD6 for the same data as (A) , normalizing to the two nearest neighbours selected from a restricted group of four autosomal probes.

laboratories testing by STS dosage with capillary electrophoresis. For STS dosage with PAGE, one laboratory recorded the sample as duplicated and one laboratory typed it as normal. H1 was reported as a partial duplication. The STS dosage method used exons from the *P0* (*MPZ*) gene as a reference control.[8] Loss of function mutations in *MPZ* can result in congenital hypomyelination, Dejerine-Sottas syndrome (OMIM #145900), or CMT1B, which are not easy to distinguish from CMT1A based on clinical signs and symptoms.[13] Therefore in this case, an alternative explanation reconciling MAPH and other results is that MPZ may be deleted, although this should result in a measured dosage ratio of 2:1, rather than 1.5:1. Attempts to resolve the true status of this DNA sample using multiplex fluorescent PCR on the remaining DNA were unsuccessful. If H1 really is duplicated at *PMP22*, as indicated by some of the tests in the Rowland *et al* study,[8] it is difficult to explain our consistent failure to detect any abnormality using a method that successfully detects all the other known duplications. Repeat sampling of this coded sample was not possible owing to the original study design.

In the case of H38, partial duplication indicated duplication of *PMP22* detected by STS dosage. Microsatellite analysis showed duplication at proximal markers but a normal pattern at distal markers, and junction fragment analysis gave a normal result. As our MAPH probes are only from the *PMP22* gene itself, all these results are consistent with duplication of the gene but not the common 1.4 Mb segment.

DISCUSSION

The measure of relative dosage for each probe in this study is based on analysis of the peak area of each probe relative to the four nearest autosomal reference probes, and normalised to results from a set of control DNA samples. The MRD6 measure integrates dosage from six probes; among the 62 samples tested in duplicate, the average of two MRD6 values in the group of 30 normal samples had a mean of 1.073 and a standard deviation of 0.101, and the group of 31 duplicated samples had a mean of 1.646 and a standard deviation of 0.183. Both these means are significantly higher ($p<0.001$ in both groups) than the expected values of 1 (normal) and 1.5 (duplicated). All samples in this blind test were normalised to known normal samples from an independent source, and one possible explanation for this systematic difference between the relative dosage of *PMP22* probes in the two sets of samples is that DNA preparation methods may have some small but reproducible effect on the efficiency of probe hybridisation to DNA on the filters.

Because MAPH data analysis uses the relative ratio of each locus compared with the four nearest autosomal framework probes, it is possible that measurement error of some framework probes adds disproportionately to the variation of some *PMP22* probes. We therefore re-analysed the data, now normalizing *PMP22* probes to a reduced framework of the least variable autosomal reference probes (probes 2q, Da, 8p, and B1: data not shown).

This time, the peak area of each probe was expressed relative to the two nearest probes from 2q, Da, 8p, and B1. It resulted in an average of MRD6 of 1.054 for normal and 1.616 for CMT1A samples. A decrease in the standard deviation (0.096 and 0.151, respectively) and a clearer discontinuity between the normal and CMT1A groups was noted (fig 4B). Apart from H39 with a MRD6 of 1.206 (which is now reported as normal, in agreement with the results of Rowland *et al*), there were no other changes of diagnosis for the samples.

One unexplained feature of our data is that despite the use of six non-overlapping probes to give a consensus report on the dosage of *PMP22*, using several probes did not give the improvement in precision expected from multiple independent measurements. Initial analyses suggest that this is the result of correlated variation in probe signal, and may be the net result of subtle but systematic variation in relative signal strengths due to small differences in hybridisation or PCR conditions. If we can identify conditions under which the probes can be more independent of such influences, we can exploit the power of multiple measurements to improve the discrimination of the test still further. However, the power of detection will be considerably weakened by mosiacism; for example, a *PMP22* duplication present in only 20% of the cells analysed would go undetected by MAPH, as it would by many other methods relying on DNA dosage to detect deletions and duplications.

ACKNOWLEDGEMENTS

We would like to thank M Cundall for help in probe preparation, E Hollox for helpful comments, I Davies for ABI gel runs and J Langdon, G Cross and A Sharif for DNA samples. We are grateful to the original laboratories that contributed samples to the CMGS study. This work was made possible by the Health Ministry of the Islamic Republic of Iran (for financial support of S M Akrami) and the Wellcome Trust (grant number 060578).

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