Advanced genome-wide screening in human genomic disorders

Knijnenburg, J.

Chapter 6

Ring chromosome formation as a novel escape mechanism in patients with inverted duplication and terminal deletion.

published in Eur J Hum Genet (2007);15(5):548-55.

Ring chromosome formation as a novel escape mechanism in patients with inverted duplication and terminal deletion

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Keywords: ring chromosome, array-CGH, inverted duplication

Abstract

Ring chromosomes are rare cytogenetic findings and are associated at phenotypic level with mental retardation and congenital abnormalities. Features specific for ring chromosome syndromes often overlap with the features of terminal deletions for the corresponding chromosomes.

Here we report a case of a ring chromosome 14 which was identified by conventional cytogenetics and shown to have a terminal deletion and an additional inverted duplication with a triplication by using large insert clone and oligo array-Comparative Genomic Hybridization (array-CGH), FISH and Multiplex Ligation-dependent Probe Amplification (MLPA).

The combination of an inverted duplication with a terminal deletion in a ring chromosome is of special interest for the described syndromes of chromosome 14. The presented findings might explain partly overlapping clinical features described in terminal deletion, duplication and ring chromosome 14 cases, since these rearrangements can be easily overlooked when performing GTG-banding only.

Furthermore, we suggest that ring chromosome formation can act as an alternative chromosome rescue next to telomere healing and capture, particularly for acrocentric chromosomes.

To our knowledge this is the first time an inverted duplication with a terminal deletion in a ring chromosome is identified and characterized using high resolution molecular karyotyping. Systematic evaluation of ring chromosomes by array-CGH might be especially useful in distinguishing cases with a duplication/deletion from those with a deletion only.

Introduction

Ring chromosomes are uncommon findings in pre- and postnatal cytogenetics. Inheritance of ring chromosomes has been reported[1-3] but the majority of ring chromosomes are *de novo*. They have been reported for all human chromosomes and are known to cause multiple congenital anomalies and mental retardation. Ring chromosomes are generally believed to result from distal breakage of the short and long arm of a chromosome and rejoining of the ends.[4] There are also reports with no apparent deletion of the telomeric ends, thus resulting in complete ring chromosomes.[5] Patients with ring chromosomes often exhibit a general overlap in phenotype, which has coined the term "ring syndrome".[6] Growth failure with no or minor anomalies is found to be the major abnormality in patients with complete ring chromosomes and is thought to be the result of cell death during development. Indeed, sister chromatid exchange during the cell cycle may cause mechanical interference of the cell division because of disruption, breakage, entangling or doubling of rings, resulting in aneuploidy and possible death of the daughter cells. In a study of 207 patients carrying a ring chromosome,[6] one fifth showed to be affected with this general ring syndrome. When both telomeric ends and coding sequences are deleted, the phenotype of the patient is in general more severe, often with specific characteristics related to the chromosome involved.

Here we report the characterization of a ring chromosome 14 containing a terminal deletion and an inverted duplication with a triplication by using molecular cytogenetic tools such as array-CGH, MLPA and FISH. The existence of a duplication with a terminal deletion in a ring chromosome similar to other duplication/deletion cases[7,8] might have clinical consequences in patients with ring chromosome 14 syndrome. This finding might explain the overlapping clinical features in patients with a ring chromosome 14 compared to patients with a terminal duplication of chromosome 14, since existing duplications in ring chromosomes can be easily overlooked at the cytogenetic level. Accordingly the large overlap in clinical features between published patients with a distal duplication and those with a distal deletion suggests that some duplication patients have an accompanying distal deletion, similar to the patient discussed in Chen et al.[9]

Here we show the importance of using combined molecular cytogenetic techniques in the characterization of chromosomal alterations, in particular in patients with ring chromosomes.

Case report

The proband is an 8-year-old girl of healthy, non-consanguineous parents. She has two sisters and two brothers, all normal. Ultrasound examination at 34 weeks of gestation showed generalized growth retardation and one umbilical artery. Birth

was at 37 weeks, birth weight was 1970 gram (p30), head circumference was 30.5 cm (<p3).
Examination at 4 months revealed craniofacial dysmorphisms namely hypertelorism, upturned nose, broad nasal bridge, malformed helices and mild micrognathia. A single palmar crease bilaterally and abnormally implanted toes were noted. At 9 months a patent ductus arteriosus was surgically closed. At present she is severely developmentally delayed. She is microcephalic (<p3), has short stature (<p3) and low weight (<p3). Growth hormone (GH) and Insulin-like Growth Factor 1 (IGF1) levels were normal. She suffers from recurrent upper and lower airway infections, eczema, scoliosis and retinitis pigmentosa. She has hypogammaglobulinemia (IgG/M and A) and normal numbers of peripheral B lymphocytes. She has sleeping difficulties, poor feeding and seizures. Informed consent was obtained from parents and patient according to routine LUMC procedure.

Materials and methods

Conventional cytogenetic analysis on GTG-banded chromosomes from cultured lymphocytes of the patient and the parents was performed according to standard techniques. From the proband and the parents 100 and 50 cells were analyzed respectively.
Fluorescence in situ hybridization (FISH) was performed according to standard protocols on metaphase chromosomes or interphase nuclei of the proband, using Cy5-ULS or D-Green-ULS labeled Whole Chromosome Painting probe (WCP) #14 (Kreatech biotechnology, Amsterdam, the Netherlands), half-YAC clone yRM2006 and Vysis® LSI® IGH/CCND1 combined probe (for 11q13 and 14q32.33, respectively) (Abbott Molecular, Hoofddorp, the Netherlands). Three BAC clones that mapped at 14q32.12 and two at 14q32.33, namely RP11-258D14, RP11-489D22, RP11-371E8, RP11-73M18 and RP11-417P24 respectively (table 1), labeled with Cy3-dUTP (GE Healthcare, Diegem, Belgium) or FITC-dUTP (Roche diagnostics, Almere, the Netherlands) were used for further confirmations.
Array-CGH was performed using ~1.0 Mb spaced whole genome large insert clone arrays, which were made available by the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk). The clones were grown, amplified and spotted as previously described.[10,11] Genomic DNA of the patient was isolated using standard techniques, and 500 ng was labeled with Cy3-dCTP (GE Healthcare, Diegem, Belgium) using the BioPrime® DNA Labeling System (Invitrogen, Breda, the Netherlands). As a reference DNA, 500 ng female human genomic DNA (Promega, Leiden, the Netherlands) was labeled using Cy5-dCTP. Hybridization and slide washing was performed without prehybridization on a HS400 hybridization station (Tecan, Giessen, the Netherlands). The arrays were scanned with a GenePix 4100A scanner (Axon Instruments, Union City, CA) and the images were processed using GenePix Pro 4.1 software. Final analysis of the intensity ratios of the

hybridized DNA was done using Microsoft Excel according to published standards.[10]

For further high resolution analysis of duplications and deletions, oligo array-CGH was performed using the Agilent Human Genome CGH Microarray Kit 244K (Agilent, Amstelveen, the Netherlands) according to the manufacturer's instructions following protocol version 4.0. Data analysis was performed with the CGH Analytics 3.4 software platform (Agilent, Amstelveen, the Netherlands).

MLPA was performed as described by White et al.[12] The selected probes were ordered from Invitrogen (Breda, the Netherlands), sequences are available as online supplementary data. Quantitative readout was done using the ABI 3730 DNA analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The accompanying Genescan 3.5 software was used for peak analysis and further downstream normalization and calculations were performed in Microsoft Excel as described before[12].

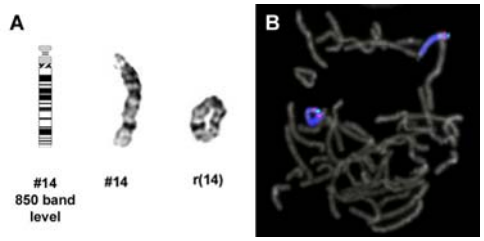

Figure 1: The ring chromosome 14 of the patient and its normal homolog. (a) GTG-banded images (b). FISH applied to a metaphase cell with BAC RP11-73M18 (red), BAC RP11-371E8 (green) and whole chromosome paint for chromosome 14 (blue), showing the inverted duplication in the ring chromosome.

Quantitative fluorescent polymerase chain reaction (QF-PCR) was performed using polymorphic short tandem repeat (STR) markers for allelotyping. Markers were chosen to cover the distal part of chromosome 14q, from 14q24.2 to 14qter.[9]

Table 1: Genomic location of BAC clones used in confirmatory FISH and genomic location of oligos around the breakpoints found with oligo array-CGH.

BAC clone	*Locus*	*Region*[a]	*Involvement*
RP11-258D14	14q32.12	92079320-92288476	in proximal side of triplication
RP11-489D22	14q32.12	92428426-92630687	in distal side of triplication
RP11-371E8	14q32.13	92565641-92758891	in proximal side of duplication
RP11-73M18	14q32.33	103217347-103382885	in distal side of duplication
RP11-417P24	14q32.33	105267358-105437117	in proximal side of deletion
Oligo	*Locus*	*Region*[a]	*Involvement*
A_14_P101212	14q32.12	91679319-91679378	in proximal side of triplication
A_16_P02972545	14q32.12	92360710-92360769	in distal side of triplication
A_14_P109278	14q32.12	92371629-92371688	in proximal side of duplication
A_16_P02989318	14q32.33	103716114-103716166	in distal side of duplication
A_16_P02989342	14q32.33	103727207-103727260	in proximal side of deletion

[a]according to Ensembl v42 database, http://www.ensembl.org/homo_sapiens/index.html

Results

Cytogenetic analysis resulted in a ring chromosome 14 in 95 of the 100 analyzed cells from the patient (figure 1a) while the remaining 5 cells showed a complex rearrangement involving endoreduplication of the ring chromosome. Both parents presented a normal karyotype. FISH using the yRM2006 probe demonstrated the absence of the subtelomeric region of 14q in the ring chromosome. In a later stage, when the proband presented with recurrent infections, the deletion was shown to encompass the IgH gene using the LSI® IGH/CCND1 combined probe, which is located at 14q32.33 at about 1 Mb from the telomere (data not shown).
Array-CGH was performed to further map the deletion. The result revealed an additional duplication of 14q32.12 to 14q32.32 (figure 2) next to the already detected deletion of 14q32.33 to 14qter. The size of the duplication was found to be 10.8 to 13.5 Mb, from BAC clone RP11-73M18 to RP11-371E8, whereas the size of the deletion was 1.1 to 3.0 Mb, from BAC clone RP11-417P24 to CTC-820M16. These findings were confirmed by FISH, whereby the duplicated clones were chosen most proximal (RP11-73M18) and distal (RP11-371E8) to the centromere. The FISH showed the duplication to be inverted (figure 1b). BAC clone RP11-417P24, located within the deleted area, was used to confirm the deletion (not shown). FISH using the same BAC probe sets showed no alteration (gain, loss or inversion) in any of the parents.

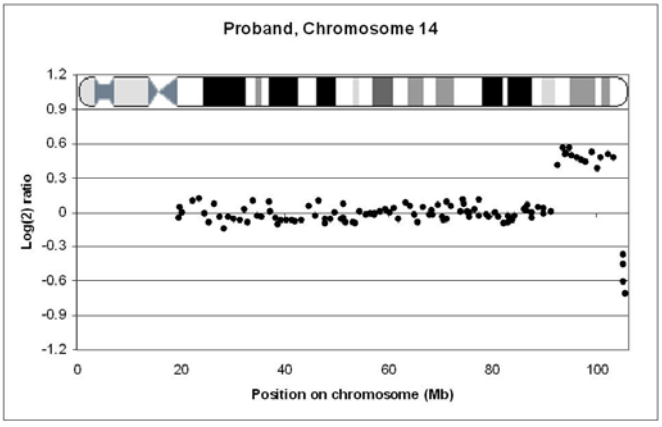

Figure 2: Array-CGH log2 ratio plot of chromosome 14 of the proband at about 1 Mb resolution, showing a distal duplication and a terminal deletion of chromosome 14 material. The dotted horizontal line at -0.3 and 0.3 represents the threshold line for deletions and duplications, respectively.

Analysis with polymorphic markers that map to the deletion, duplication and a normal part of the chromosome, proved the duplication to be intrachromosomal. The marker analysis showed a duplication of one allele of markers D14S557 and D14S543 (Table 2). Parental analysis revealed that the ring chromosome is of paternal origin.
Additional high resolution oligo array-CGH confirmed the previous findings, the size of the amplified region was estimated to be 11.344 Mb and the size of the deletion was estimated to be 2.641 Mb. The genomic locations of the oligos

Table 2: Location of short tandem repeat (STR) markers and genotypic results in the proband and her parents.

Markers	*Locus*	*Region*[a]	*mother*	*father*	*proband*
D14S620	14q24.2	72401116-72401231	114,114	114,118	114,118
D14S739	14q31.1	81336367-81336556	184,184	176,184	176,184
D14S616	14q31.3	84263655-84263874	211,215	223,223	211,223
D14S128	14q31.3	85450372-85450704	336,363	332,344	332,336
D14S617	14q32.12	91272543-91272683	139,161	161,165	139,165
D14S557	14q32.32	102189847-102190162	304,320	275,287	287,287,304
D14S543	14q32.33	103658598-103658852	244,252	241,249	249,249,252

[a] according to Ensembl v42 database, http://www.ensembl.org/homo_sapiens/index.html

around the breakpoint are found in table 1. Interestingly, this assay revealed a triplicated 681 kb region in the proximal duplication region, belonging to band 14q32.12 (figure 3). A confirmatory MLPA test using probes designed for all altered regions (triplicated, duplicated, deleted and normal) proved the presence of all alterations, including the triplication of the region in band 14q32.12 (figure 4) and it showed that the parents of the proband have a normal copy number for all tested regions. FISH using BAC's RP11-258D14 and RP11-489D22 showed that the triplicated region is located within the ring, at the distal side of the rearranged q-arm (not shown).

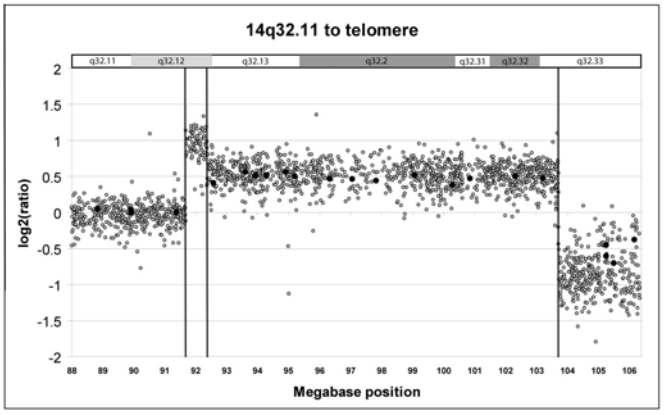

Figure 3: A combined display of the oligo array-CGH (open circles) and the ± 1 Mb resolution large insert clone array-CGH (closed circles), log2 ratio plots of the chromosome 14q32.11-qter region of both experiments on the proband are shown. The oligo array shows a triplication, a duplication and a deletion of the distal region of chromosome 14. The clones of the 1 Mb array fall just adjacent to the triplication.

Discussion

Using the combination of FISH and array-CGH in genetic analysis has demonstrated that seemingly simple rearrangements are often more complex than defined by GTG-banding.[10,13,14] After conventional cytogenetic screening (figure 1a) the karyotype of the proband was described as 46,XX,r(14)(p12q32). To further map the breakpoint, array-CGH was performed with a resolution of about 1 Mb (figure 2). Next to the expected deletion of band 14q32.33 an additional duplication of 14q32.12 to 14q32.32 was revealed. FISH using the first

and the last duplicated BAC clone confirmed this finding and showed the duplication to be inverted (figure 1b), which originated from one paternal chromosome based on STR-allelotyping (table 1).

An inverted duplication associated with a terminal deletion was first described for distal chromosome 8p,[7,8] followed by 1q,[15] 4q,[16] 15q,[17] and, more recently, also for chromosome 14q.[9] A mechanism for the formation of inverted duplications associated with terminal deletions has been described both for intrachromosomal duplications, based on normal parental chromosomes[18] and for intra- and interchromosomal duplications based on a parental inversion carrier.[19] These proposed mechanisms assume that an intrachromosomal U-type recombination during meiosis I, or a loop formation combined with one or two recombination events between homologous alleles, has occurred. The consequential recombinant dicentric chromosome would be deleted beyond the distal recombination site. At meiosis II, the two linked chromatids can segregate to the opposite poles resulting in a breakage between the two centromeres. If this breakage occurs asymmetrically, the two resulting recombinant products are a derivative with an inverted duplication and a deleted 14q derivative.

When chromosomal rearrangements take place, either constitutional or tumorigenic, broken chromosome ends need to be stabilized to prevent end-to-end fusions and exonucleolytic degradation. Telomere healing can be accomplished by addition of human telomeric tandem repeat sequence to broken chromosome ends[20] or by telomere capture, which in fact is actually subtelomeric translocation to the broken chromosome end, resulting in an extra duplicated subtelomeric region. This latter mechanism is proven in melanoma and other cancer cell lines and in irradiated lymphoblastoid and fibroblast cells.[21] Examples of constitutional telomere capture are less common and is only reported a few times.[22,23] Among the several inverted duplication/deletion events reported,[7-9,15-19,23] in a single case the broken chromosome was proven to be repaired with telomere capture.[23]

On the contrary, in our case the healing of the broken end may have been mediated by ring formation. In an acrocentric chromosome, an additional break involving the short arm probably does not lead to an additional loss of coding sequence and consequently to impaired cell proliferation. The fact that ring chromosome formation in 47% of the reported cases[6] involves acrocentric chromosomes, supports this notion.

If the recombination were based on non allelic homologous recombination (NAHR), then a fragment with a normal copy number would still be present between two recombined low copy repeats, between the duplicated and the deleted region at 14q32.32-14q32.33. Since, neither the 1 Mb BAC array nor the STR-allelotyping was conclusive to locate a possible fragment with a normal copy number between the duplicated and deleted region, an oligo array was performed. The 244k Agilent oligo array platform was chosen because between the distal duplicated and proximal deleted BAC clones there were 271 oligo

reporters available, while for example in the 500k SNP based chips from Affymetrix 120 SNP elements were present with an uneven coverage of the region of interest, including some gaps of 150 kb.

Table 3: Comparison of the clinical features in published cases with distal chromosome 14 alterations and the presented case.

	Van Karnebeek et al.[25]				Chen et al. [9a]			Our proband
	Linear 14q deletions q32-distal	(in %)	Ring 14 deletions q32-distal	(in %)	14q duplications q31-qter	(in %)	Inv dup/del case	Inv dup/del ring case
No. of cases	12		20		9			
Gender (males)	3		8		5			
Mental retardation	11/11	100	20/20	100	7/7	100	+	+
Seizures	1/10	10	19/20	95				+
Hypotonia	8/12	67	11/19	58	5/9	56	+	+
Prenatal growth retardation	5/12	42	7/16	44				+
Postnatal growth retardation	5/12	42	9/17	53	7/8	88	+	+
Microcephaly	4/12	33	14/18	78	6/9	67	+	+
Dolichocephaly	3/8	38	5/12	42				
High forehead	10/10	100	7/12	58				
Prominent forehead	7/10	70	9/12	75				
Hypertelorism	3/10	30	7/15	47	7/9	78	+	+
Strabismus	3/9	33	4/15	27	1/7	14	-	-
Blepharophimosis	6/10	60	10/13	77	0/7	0	+	+
Ptosis	4/11	36	2/14	14	0/7	0	-	-
Downslanting palpebral fissures	6/11	55	8/13	62	7/9	78	+	-
Epi-/telecanthi	8/12	67	12/17	71	2/9	22	+	-
Broad/flat nasal bridge	10/11	91	10/13	77				+
Anteverted nares	2/9	22	5/12	42				+
Dysmorphic nose					6/9	67	+	+
Short bulbous nose	5/10	50	6/11	55				-
Long philtrum	4/9	44	4/11	36				-
Broad philtrum	8/10	80	8/11	73				-
Thin upper lip	6/8	75	5/11	45				
Small, fish shaped mouth	6/10	60	6/13	46				-
Highly arched palate	9/10	90	7/13	54			-	-
Abnormal dentition	2/6	33	2/10	20				
Low set ears	3/9	33	7/8	88				-
Malformed helices	5/8	63	3/8	38	7/9	78	+	+
Micrognathia	6/11	55	5/12	42	8/9	89	-	+
Pointed chin	4/10	40	1/12	8				-
Short neck	1/6	17	7/14	50				
Webbed neck	2/6	33	1/11	9				-
Congenital heart defect	3/9	33	1/15	7	6/9	67	+	+
Single palmar crease	6/7	86	1/15	7				+
Brachydactyly	0/3	0	3/9	33				-
Clinodactyly	2/7	29	1/8	13	7/9	78	+	-
Tapering fingers/arachnodactyly	2/5	40	2/9	22				
Retinitis pigmentosa	0/5	0	8/16	50				+
Scoliosis								+

[a] and references herein.

Despite the fact that the oligo array did not detect a region of normal copy number between the duplicated and the deleted fragment, the region of the distal breakpoint could be narrowed down to ~11 kb.
There was no evidence for the presence of LCR's in this interval (Ensembl, release 42), but this region does encompass a 2.8 kb chained self alignment fragment having homology within its own region. Possibly this region can cause hairpin or loop formation and can mediate recombination or a double strand break, eventually leading to the U-type of translocation.

Moreover, the oligo array showed an extra copy number gain of the first 600 kb of the duplicated region (figure 3), at 14q32.12, which was confirmed with MLPA (figure 4). Based on FISH results with a BAC probe from this region, it was found that the extra 600 kb fragment is inserted at the distal side of the chromosome, the part that is fused to the p-arm side of the centromere. In complex chromosome rearrangements, often more breaks are found than actually needed to explain the rearrangements on cytogenetic level.[10,13,14] The extra amplification of this fragment at the distal side of the chromosome is probably a secondary event, following the inverted duplication and the distal break of the dicentric chromosome, illustrating the struggle to rescue the chromosome. The finding of this additional triplication stresses the importance of introducing high resolution techniques in investigating genetic aberrations in patients.
Due to the large overlap in the clinical features of cases with rearrangements within the distal region of chromosome 14 (table 3), ring chromosome 14, 14q deletion, and chromosome 14 distal duplication syndromes should be discussed together.

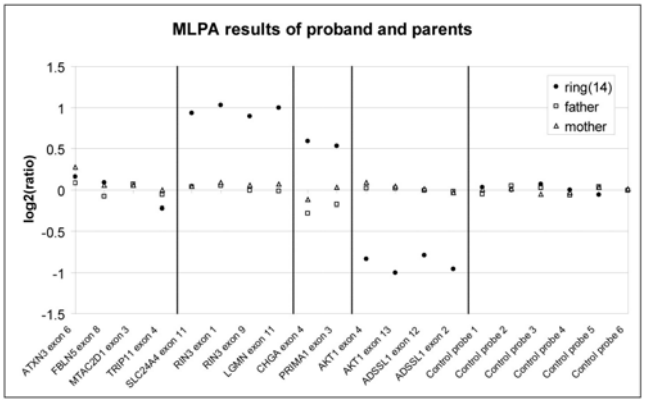

Figure 4: Log 2 ratio plot of the confirmatory MLPA. The proband (closed circles) shows triplication, duplication and deletion of the expected probes. Both parents show no copy number change in the tested regions. Three control probes were tested in duplo (control probe 1-6) and are located in the EXT1 gene on chromosome 8q24.11, in the CREBBP gene on 16p13.3 and in the p300 gene on chromosome 22q13.2.

The ring chromosome 14 syndrome was delineated by Schmidt et al.,[24] and it was further described by van Karnebeek et al.[25] A distal duplication of chromosome 14 results in a variable clinical picture that is mainly depending on the size of the duplication.[9,26] Main features that are found in distal duplications of 14q31→qter are mental and growth retardation, microcephaly, hypertelorism, abnormal ears, micrognathia and congenital heart defects. In the case of Chen et al., an additional terminal deletion was demonstrated using FISH, next to the duplication. The small terminal deletion, which in itself can lead to a severe phenotype,[25] or an additional duplication in a ring chromosome, is sometimes only detectable by molecular techniques such as FISH and array-CGH. It emphasizes the significance of high resolution molecular karyotyping for the establishment of accurate phenotype/genotype correlations.

Acknowledgements: We appreciate the supervision of Drs E. Aten in the MLPA analysis.

Supplementary information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)

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