**ABSTRACT**

Deletions involving 3p are believed to be typical for conventional (clear cell) renal cell carcinoma (cRCC), with confirmed and suspected targets being the VHL and FHIT tumor suppressor genes, respectively. By contrast, 3p deletions are felt to be rare in papillary RCC (pRCC) and chromophobe RCC (chRCC); however, this belief is based on relatively scant data. In particular, 3p14.2 deletions, possibly resulting in FHIT inactivation, have been rarely studied in pRCC or chRCC even though they may be relevant in early renal tumorigenesis. We therefore examined 3p deletion rates and patterns in pRCC and chRCC with particular attention to 3p14.2. We examined 16 chRCCs and 27 pRCCs for loss of heterozygosity (LOH) at 3p25–26 and 3p14.2 using 13 well-mapped microsatellite markers. Those pRCC with LOH at 3p25–26 were also screened for FHIT gene mutations. The results were correlated with tumor histology and patient outcome and compared with data we had obtained previously on cRCC. We found similar overall 3p LOH rates in pRCC (59%), chRCC (86.6%), and cRCC (75.8%). In pRCC and chRCC, LOH at 3p25–26 was more common than at 3p14.2, whereas the converse was true for cRCC. In the pRCC with 3p25–26 LOH, we confirmed that this was not associated with mutations of the VHl gene. At 3p14.2, LOH rates of pRCC were lower than those of cRCC and chRCC (p < 0.02). All morphotypes showed a predominately interstitial LOH pattern, which was most pronounced in the 3p14.2 region in cRCC. 3p LOH in chRCC was associated with improved patient outcome, mirroring our previous cRCC data. We conclude that 3p LOH is a universal phenomenon in RCC, but has different underlying mechanisms, molecular targets, and implications in the different morphotypes, although FHIT inactivation may play a role in both cRCC and chRCC tumorigenesis.

**INTRODUCTION**

RCC is not a single tumor type, but consists of a variety of different morphotypes. The most frequently encountered RCC subtype is cRCC, representing 70% of all adult primary renal tumors. The second most common morphotype is pRCC, accounting for 10–15% of malignancies, and ~5% of renal cancers are chRCCs. The remainder is made up of collecting duct carcinomas and unclassifiable tumors (1–4).

Specific genetic abnormalities have been associated with each of the three major morphotypes of cRCC, pRCC, and chRCC (1–3), leading some authors to propose the usage of molecular testing in the subtyping of renal epithelial tumors (5).

Genomic deletions involving the short arm of chromosome 3 are considered to be a typical feature of cRCC, and the VHL tumor suppressor gene at 3p25–26 is believed to be the primary target of these genetic deletions. In most cRCCs, VHL is found to be inactivated by a combination of allelic deletion and either mutation or, less commonly, hypermethylation (3). However, on the basis of LOH studies, it is suspected that additional cRCC tumor suppressor genes may reside on 3p. The FHIT gene at 3p14.2 is an increasingly attractive candidate, and it has been suggested that another, as yet unidentified, tumor suppressor gene may reside at 3p12 (6, 7). pRCCs have been reported to show trisomy 7 or 17, loss of Y chromosome, (3, 8) and, in familial cases, mutations in the MET oncogene at 7q31 (3, 9), whereas 3p deletions are believed to be rare (3, 10), and mutations of the VHL gene have not been reported (11, 12). Similarly, 3p deletions are also thought to occur only in about 25% of chRCCs (12–14), which are otherwise characterized by multiple, large chromosomal deletions involving chromosomes 1, 2, 6, 10, 13, and 17 (13–15). These observations have led to the impression that in RCCs there are close genotype-phenotype correlations, which are possibly of diagnostic and prognostic significance. However, on closer inspection, the common perception of morphotype-specific tumor-genetic alterations in the major RCC morphotypes, particularly with regard to chromosome 3 changes, is based on relatively scant data for pRCC and chRCC. The numbers of pRCCs and chRCCs included in various studies have often been small, with tumors being incompletely characterized in histopathological terms. Moreover, most of the observations are based on cytogenetic or LOH studies using a limited number of markers (12–18). Either of these methodologies is likely to reveal only large chromosomal deletions, not interstitial losses. Moreover, cytogenetic studies will also fail to detect nondisjunctional events, with subsequent chromosomal reduplication.

We have recently shown that interstitial LOH patterns predominate in cRCC, and that FHIT locus LOH exceeds VHL locus LOH, representing a potentially early tumorigenic event (19). Previously, Shridhar et al. (20), using multiple 3p14.2 probes, have found high 3p LOH rates in non-cRCCs, including pRCC and chRCC. The numbers of pRCCs and chRCCs included in various studies have often been small, with tumors being incompletely characterized in histopathological terms. Moreover, most of the observations are based on cytogenetic or LOH studies using a limited number of markers (12–18). Either of these methodologies is likely to reveal only large chromosomal deletions, not interstitial losses. Moreover, cytogenetic studies will also fail to detect nondisjunctional events, with subsequent chromosomal reduplication.

**MATERIALS AND METHODS**

**Tumor Samples.** All studies were approved by the Wellington Ethics Committee. The tumor specimens were obtained from the archives of Wellington Hospital, Wellington, New Zealand, and the Klinikum Wuppertal, Wuppertal, Germany. From these sources, we identified 27 pRCCs and 16 chRCCs from patients for whom a minimum of 5-year postoperative follow-up data were available. The sections from each case were reviewed and the tumors were staged according to the International Union Against Cancer TNM system and graded according to the Fuhrman classification (21, 22). In addition, pRCC were subclassified as types 1 or 2 on the basis of papillary microarchitecture (4).

**DNA Extraction.** Paraffin-embedded blocks of formalin-fixed tissue from each case were cut at 10 μm thickness. Histologically representative, unstained sections from each of the tumors were microdissected into neoplastic compartments containing at least 80% tumor cells (typically >90%) and control compartments containing normal renal tissue without any microscopic evidence of tumor. After microdissection, the samples were deparaffinized by
successive xylene and ethanol washes and incubated in 150 μl of DNA extraction buffer [2.7 μg/μl proteinase K, 100 mM Tris, and 2 mM EDTA (pH 8)] at 55°C for 48 h. Additional proteinase K (2.7 μg/μl) was added after 12 h. After heat inactivation of proteinase K, aliquots of the digests were used directly for PCR.

**Microsatellite LOH Analysis.** LOH analysis was performed by paired normal-tumor microsatellite PCR. Fig. 1 shows the localization of the markers used in our study, ordered on the basis of the Genome Database, 4 the Genetic Location Database, 5 the Cooperative Human Linkage Center database, 6 and the dbSTS database. 7

PCRs contained 2.5 μl of digested sample solution, 200 μM of all four deoxynucleotide triphosphates, 500 nM each of forward and reverse primers, 0.02 units/μl Taq polymerase (Roche Diagnostics N. Z. Ltd., Auckland, New Zealand), 1X PCR buffer (Roche Diagnostics N.Z. Ltd.), and 1.5 mM Mg2+ in total reaction volumes of 25 μl. Either forward or reverse primers were labeled with 6-FAM or HEX (Sigma Chemical Co.-Genosys, Sydney, Australia). PCR conditions were optimized as necessary.

For LOH analysis, PCR products were diluted between 1:2 and 1:8, combined with size-standard 500-TAMRA (Applied Biosystems, Melbourne, Australia), and deionized formamide/loading dye; denatured at 95°C for 3 min, quenched on ice, loaded on denaturing 4% polyacrylamide/8 M urea gels, and electrophoresed on an automated gene sequencer 377 (Applied Biosystems).

Fluorescent gel data were analyzed with GeneScan 2.1 software (Applied Biosystems). For each informative tumor-control pair of reactions (two alleles visible in control samples), an allelic imbalance ratio was calculated from the fluorescent peak allele heights in tumor and corresponding normal control samples as follows: control-allele1:control-allele 2/tumor allele1:tumor allele 2. LOH was defined as an allelic imbalance ratio of <0.6 or >1.67. This corresponded to allelic loss in at least 50% of tumor cells in an 80% pure tumor sample. In addition, we defined complete allelic loss/LOH as allelic loss in all tumor cells, corresponding to an allelic imbalance ratio of <0.2 or >5 in an 80% pure tumor sample. Breakpoints were defined as regions between immediately adjacent markers, with or without LOH.

**SSCP Mutation Screening of VHL in pRCCs.** For all pRCC specimens with 3p25–26 LOH, the three translated exons of VHL were PCR-amplified separately using previously published primers and conditions which allow coverage of >95% of the coding region of the gene (11). PCR products were diluted between 1:2 and 1:4, combined with size-standard 500-TAMRA (Applied Biosystems) and deionized formamide/loading dye; denatured at 95°C for 2 min, quenched on ice, loaded on 0.5% mutation detection enhancement gels (BMA, Rockland, ME), and electrophoresed for 14 h at 25°C on a 377 automated gene sequencer (Applied Biosystems), using a recirculating refrigerated water bath (Neslab Instruments, Inc., Newington, New Hampshire) for cooling. In addition to the tumor specimens, each gel contained several lanes with wild-type control PCR products.

After electrophoresis, the fluorescent gel data were analyzed with GeneScan 2.1 software (Applied Biosystems). Lanes were aligned to each other and the wild-type control lanes using the in-lane size standards as guides, and the fluorescent PCR products were inspected for differential migration as compared with the wild-type control products.

**Statistical Analysis and Genotype-Phenotype Comparisons.** Kaplan-Meier analysis, using the log-rank test for significance testing, was used to compare the effect of the presence or absence of LOH at 3p, 3p25–26, and 3p14.2 on patient survival.

LOH frequencies within the examined regions and at individual loci were compared with the average LOH rates for the morphotypes using contingency tables and χ² or Fisher’s exact test for significance testing.

Comparison of LOH patterns of pRCC and chRCC with each other and with cRCC was also based on contingency tables. The raw data for cRCC were obtained from our previous study, which had used identical methodology except for the utilization of radioactive labeling and the detection of microsatellite markers rather than fluorescent technology (19).

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5 Internet address: http://cedar.genetics.soton.ac.uk/public_html.
6 Internet address: http://www.chlc.org/.

**RESULTS**

**chRCC.** The average patient age at diagnosis was 50.2 years; eight patients were female and eight were male. The 5-year mortality rate was 56.2%, with a median survival interval of 54 months. One tumor was classified as TNM stage 1, seven were stage 2, six were stage 3, and two were stage 4. Division by Fuhrman grade resulted in 10 grade 2, 5 grade 3, and 1 grade 4 tumors. D3S2977 was not informative in all cases. The other 12 markers were informative in a mean of 75.85% of cases. One specimen was not informative at all loci. 3p LOH was detected in 13 of 15 informative specimens (86.6%), whereas complete allelic loss (allelic imbalance ratio of <0.2 or >5) occurred in 7 of 15 specimens (46.7%). LOH at 3p 25–26 occurred in 10 of 14 cases (71.4%), which were informative in this region, whereas 10 of 15 tumors (66.6%), which were informative at 3p14.2, displayed LOH (Fig. 2). The figures for complete allelic loss were 3 out 14 (21.4%) for 3p25–26 and 4 of 15 (26.7%) for 3p14.2.

The average rate of LOH per marker was 34.8%. At 3p25–26 this was 38.5%, whereas at 3p14.2 it was 27.5%. The most frequent sites (26.7%) for 3p14.2.

**pRCC.** The average patient age at diagnosis was 63.4 years; 8 patients were female and 19 were male. The overall 5-year mortality...
rate was 50%, with a median survival interval of 60 months. Eleven tumors were TNM stage 1, eight were stage 2, seven were stage 3, and one tumor was stage 4. Division by Fuhrman grade resulted in 16 grade 2 and 11 grade 3 tumors. No tumors were graded as grade 4. Sixteen specimens were classified as type 1 and 11 as type 2 tumors.

D3S2977 again was not informative in all cases. The other 12 markers were informative in a mean of 70.3% of cases. All specimens were informative for at least one locus. 3p LOH was detected in 16 of 27 specimens (59%). Complete allelic loss occurred in 7 of 27 samples (25.9%). LOH at 3p 25–26 occurred in 14 of 26 samples, which were informative in this region (53.8%) significantly more frequently ($P < 0.01$) than at 3p14.2 (11 of 27; 40.7%; Fig. 2). The figures for complete allelic loss were 6 of 26 (23.1%) for 3p25–26 and 5 of 27 (18.5%) for 3p14.2.

The average rate of LOH for the 12 informative markers was 24.3%, with the rates for both 3p25–26 (27.73%), and 3p14.2 (19.3%) not differing significantly from that figure. The most frequent sites of LOH were D3S1038, (61%; $P < 0.001$) within 3p25–26 and D3S1300 at 3p14.2 (42%; $P < 0.025$). In the 3p14.2 region, we observed a total of eight break points in four tumors: two between D3S4103 and D3S1300, two between D3S1300 and D3S4260, two between D3S4260 and D3S1480, and two between D3S1480 and D3S1481. There were no differences in LOH rates or patterns between the two pRCC subtypes.

Whether a tumor displayed 3p LOH or not had no influence on cause-specific patient survival (Fig. 3).

Because of the high LOH rates observed at 3p25–26 in our pRCC specimens, we considered the possibility that in our cohort of pRCC, VHL may be the target of these deletion events. We therefore performed single-strand conformational polymorphism mutation screening of the three translated VHL exons in the 14 pRCC specimens, which had displayed 3p25–26 LOH. This failed to reveal any possible VHL mutations.

Comparisons between pRCC, chRCC, and cRCC. The total 3p LOH rates, as well as the LOH rates at 3p25–26, did not differ statistically between cRCC, pRCC, and chRCC. However, chRCC and pRCC displayed higher rates of LOH at 3p25–26 than at 3p14.2, whereas the converse was true for cRCC. At 3p14.2, the LOH rates of cRCC and chRCC also did not differ from each other; but for pRCC, LOH within this region occurred significantly less commonly than in the two other morphotypes ($P < 0.02$; Fig. 2).

The LOH rates per marker differed only slightly between the three morphotypes, the only significant difference being that LOH at D3S1560 ($P < 0.0045$) and at D3S1038 ($P < 0.001$), both within 3p25–26, was more common in chRCC than in the other morphotypes.

With regards to LOH patterns, all morphotypes showed a predominately interstitial LOH pattern, but this was most pronounced in cRCC, particularly within the 3p14.2 region. In cRCC, 131 breakpoints were observed within the 3p14.2 region in 57 (of 87) tumors, far outnumbering the corresponding figures in the other two morphotypes (see above), both in absolute terms and in breakpoints/tumor ($P < 0.001$). When the criteria for complete allelic loss/LOH (allelic imbalance ratio of $<0.2$ or $>5$) were applied to the data, the LOH patterns in all morphotypes remained largely interstitial, although the LOH rates per marker and per tumor type were lower when using these stricter criteria.

The presence or absence of 3p LOH was correlated with cause-specific patient survival only in chRCC (Fig. 3). However, as reported previously, a higher proportion of cRCC tumors with 3p LOH were of lower grade than those without 3p LOH (19).

DISCUSSION

This study has shown that both pRCC and chRCC display 3p LOH at the same overall rates as cRCC, but that LOH patterns differ between the morphotypes. In addition, for chRCC, our data reinforce our previous observations in cRCC of a possible link between 3p LOH and less aggressive tumor behavior, whereas no such link was evident for pRCC.

Conventional wisdom suggests that 3p LOH is a defining feature of cRCC, intimately linked with its pathogenesis via VHL inactivation, whereas this is reported to occur only rarely in pRCC or chRCC. Some authors have proposed the use of the presence or absence of 3p LOH as a molecular differential diagnostic tool in the pathological assessment of RCC (5). However, such assertions are largely based on cytogenetic or low-resolution LOH data obtained on relatively small numbers of pRCCs or chRCCs (3, 12–18, 20). In particular for chRCCs, there is a growing body of evidence that suggests that these early observations may not be entirely correct. In 1992, Kovacs et al.
other authors since have reported 3p LOH rates of (14) reported 3p LOH in 5 of 9 chRCC specimens studied, and several other authors since have reported 3p LOH rates of ~25% in chRCC (12–14), although LOH rates of >80%, as observed in our study, have not been reported.

By contrast, in pRCC, 3p LOH is still considered an extreme rarity (3), despite some recent observations suggesting that it may occur in a minority of tumors (17, 18, 20). Our data support these latter findings and enlarge on them, suggesting that the traditional viewpoint needs to be revised. In our series of histologically well characterized tumors, 3p LOH occurred in 59% of specimens, being about equal in frequency to that found in cRCC and chRCC. Moreover, 3p LOH of a similar pattern occurred in both type 1 and type 2 pRCCs, suggesting that it is not limited to a particular pRCC subtype.

The failure of many previous studies to identify significant 3p LOH rates in chRCC and, more prominently, pRCC, could be attributable to several factors. First of all, it may be a result of selection bias or random variation. This can lead to substantial differences between different retrospective, nonblinded studies. Secondly, much of the early work on the genetics of RCC involved cytogenetic studies of tumor specimens or LOH studies using a limited number of markers. Cytogenetic studies will not identify chromosomal nondisjunctional events with subsequent reduplication of the remaining chromosome. Furthermore, both methods are unlikely to detect small genomic deletions. Indeed, most of the more recent studies, which do report significant 3p LOH rates in chRCC or pRCC, use much larger numbers of markers, suggesting interstitial LOH is occurring in these morphotypes. These results are supported by our own findings, which show that apparently interstitial LOH patterns predominate over contiguous losses. Even when applying extremely strict LOH criteria to our data, i.e., allelic loss in all tumor cells, rather than the more commonly used criterion of allelic loss in at least 50% of tumor cells, interstitial LOH patterns are equally common or more common than contiguous losses. This makes it unlikely that the observed LOH patterns represent artifacts relating to varying degrees of intratumor genetic heterogeneity or marker-choice. However, only additional testing with many additional markers or alternative techniques would unequivocally prove that the interstitial patterns are not caused by intrachromosomal rearrangements. In the context of interstitial LOH, the choice of markers may also be important, as some markers seem to be more likely to exhibit LOH than others. An example of this phenomenon is D3S1300, which we and others have found to display particularly high rates of LOH, possibly as a result of being located within the fragile chromosome 3 site B (FRA3B ) (19, 20). Finally, inaccurate histopathological diagnosis can contribute to differing results. The results of any molecular study will be unreliable if significant proportions of misclassified tumors are included. This problem is particularly relevant with regards to chRCC, where it is now realized that there is a diagnostic overlap between benign oncocytomas and eosinophilic chRCCs when tumors are characterized by light microscopy (23). Such misdiagnosis may not be rare, as suggested by the fact that some series show a (sometimes substantially) more favorable prognosis for chRCC than for cRCC, whereas others, including our present study, find similar cause-specific mortality rates for both morphotypes (24, 25). In combination, these factors may have led to a substantial underestimation of 3p LOH rates in chRCC and pRCC.

Our results have obvious implications for the potential use of 3p LOH studies in the molecular differential diagnosis of renal tumors. In addition, they raise the question of the pathogenic relevance of 3p LOH in RCC.

In cRCC, there is overwhelming evidence to support the suggestion that 3p LOH leads to genetic deletions or rearrangements that inactivate one allele of the VHL tumor suppressor gene, whereas the other allele is inactivated by mutation or, less commonly, hypermethylation (11, 12, 26). LOH at 3p14.2 or 3p12 may inactivate additional tumor suppressor genes. A strong candidate for this is FHIT at 3p14.2, a common target of deletions in cRCC (19), with probable pathogenic significance because expression of FHIT protein is lost in the majority of cRCC (27–29). By contrast, almost all pRCCs and ~80% of chRCCs seem to retain FHIT protein expression (28, 29).

In our study, chRCC and pRCC showed similar LOH rates in the VHL, 3p25–26, to cRCC. Particularly in the pRCCs, LOH at 3p25–26 was, in fact, relatively more common than LOH at 3p14.2. Moreover, the region affected by LOH included markers which are very close to the VHL gene locus. This suggested to us that, in our group of pRCC specimens, VHL may be inactivated. However, mutation screening of the 14 pRCC specimens, which had displayed LOH at 3p25–26, failed to identify any mutations in the three translated exons of the VHL gene. This is in agreement with previous mutation screening studies of chRCC and pRCC, which have only rarely shown evidence of VHL mutations (3, 11, 12, 30). Because the other nondeletional mechanism of VHL inactivation, hypermethylation, accounts for a much smaller percentage of VHL inactivation than mutation, this suggests that VHL is not the target of biallelic inactivation in our pRCC specimens. Immunohistochemistry or Western blot studies, which for some tumor suppressor genes, such as FHIT, can be helpful in confirming such conclusions, unfortunately do not seem to be helpful in assessing VHL inactivation. The VHL protein is generally expressed in tumor tissues, presumably in an inactive form, even when a classic tumor suppressor gene somatic genetic “double hit” has been confirmed (31, 32). Antibodies against the whole VHL protein (rather than specific domains only), which might demonstrate reduced levels of functional VHL protein, have not been produced thus far.

The fact that VHL does not seem to be the target of the observed subtelomeric 3p LOH in our tumors suggests that these deletions may either be a common nonspecific event or that one or several additional tumor suppressor genes reside in this region. Another possibility is that, in chRCC or pRCC, 3p LOH does not actually reflect allelic deletion but amplification, resulting in oncogene activation rather than tumor suppressor gene inactivation. Genetic amplification can lead to allelic imbalance on microsatellite PCR similar to genetic deletion. However, this seems an unlikely scenario for chRCC, which is generally characterized by multiple genetic deletions (13, 15). By contrast, in pRCC, chromosome polysomies and genetic amplifications are frequently observed (3, 8, 9), and it has been suggested that 20% of pRCCs may harbor genetic amplifications involving 3p (33).

Potentially fundamental differences between the three morphotypes become apparent when 3p14.2 LOH rates and breakpoint frequencies are compared. Within 3p14.2, pRCC exhibited significantly lower LOH rates than cRCC or chRCC. Both chRCC and pRCC displayed relatively more 3p25–26 LOH than 3p14.2 LOH, whereas the converse was true for cRCC. In addition, 3p14.2 breakpoints occurred in a larger proportion of tumors and at higher rates per tumor in cRCC than in chRCC or pRCC.

These findings suggest that the molecular events underlying 3p14.2 LOH also differ between the morphotypes, with chRCC displaying more similarities to cRCC than to pRCC. The latter is supported by our finding of reduced cause-specific mortality in chRCC patients with 3p LOH. These survival data mirror our previous observations in cRCC, where 3p LOH and 3p14.2 LOH were associated with lower tumor grades (but not with better survival). By contrast, no such mechanism seems to exist for pRCCs, suggesting, that the relatively uncommon 3p14.2 LOH in this morphotype may be an epiphenomenon.

Possible explanations for the favorable prognostic impact of 3p
LOH in cRCC and chRCC include the possibility that 3p14.2 LOH is an early tumorigenic event that is associated with slow tumor progression.

Neoplasms that acquire malignant potential via alternative routes behave more aggressively, and inactivation of FHIT as an early tumorigenic event could be the underlying mechanism. There is marked heterogeneity in the potency of FHIT to act as a tumor suppressor in RCC cells, and it has been suggested that FHIT inactivation on its own may be only a relatively weak tumorigenic event, requiring additional genetic changes for tumorigenesis (34, 35). Such a model would be in accord with our chRCC and cRCC prognostic data, with early FHIT inactivation propelling a cell on a relatively insidious tumorigenic pathway, whereas other initiating events result in a higher early malignant potential. However, there is also evidence which suggests that FHIT may not be a common target in chRCC.

Published data on FHIT protein expression suggest that, whereas most cRCCs lose FHIT protein expression, pRCCs generally retain it (27–29), and chRCCs lose it in only 20% of cases. Our breakpoint data are consistent with these observations, showing multiple, potentially FHIT gene-disruptive events in the majority of cRCCs but only in a small number of chRCCs. In view of these findings, FHIT inactivation may not be the commonly prognostically relevant early event in cRCC and chRCC, although such a possibility cannot be excluded. In particular, a subset of chRCC may share early FHIT inactivation with cRCC, whereas in the larger proportion of chRCCs, the mechanisms of 3p14.2 breakage may be different.

In conclusion, it seems that 3p LOH is an almost universal phenomenon in all three main RCC morphotypes. However, it also seems that underlying mechanisms, molecular targets, and clinical implications of the 3p LOH differ between the morphotypes. Interesting new questions and directions for research emerge from the association of these observations, showing multiple, potentially FHIT gene-disruptive events in the majority of cRCCs but only in a small number of chRCCs.
VHL and FHIT Locus Loss of Heterozygosity Is Common in All Renal Cancer Morphotypes But Differs in Pattern and Prognostic Significance

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