Gain of 1q is Associated with Inferior Event-Free and Overall Survival in Favorable Histology Wilms Tumor: A Report from the Children's Oncology Group

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Abstract

Background—Wilms tumor is the most common childhood renal tumor. While the majority of patients with favorable histology Wilms Tumor (FHWT) have good outcomes, some patients still experience recurrence and death from disease. This study's goal was to determine if tumor-specific chromosome 1q gain is associated with event-free (EFS) and overall survival (OS) in FHWT.

Methods—Unilateral FHWT samples were obtained from patients enrolled on National Wilms Tumor Study-4 and Pediatric Oncology Group 9046, "A Molecular Genetic analysis of Wilms Tumor." 1q gain, 1p loss, and 16q loss were determined using multiplex ligation-dependent probe amplification (MLPA).

Results—The eight-year EFS was 87% (95% CI 82%, 91%) for the entire cohort of 212 patients. Tumors of 58/212 patients (27%) displayed 1q gain. A strong relationship between 1q gain and 1p/16q loss was observed. The eight-year EFS was 76% (95% CI 63%, 85%) for those with 1q gain and 93% (95% CI 87%, 96%) for those lacking 1q gain (p=0.0024). The eight-year OS was 89% (95% CI 78%, 95%) for those with 1q gain, and 98% (95% CI 94%, 99%) for those lacking 1q gain (p=0.0075). Gain of 1q did not correlate with disease stage (p=0.16). After stratification for stage, 1q gain was associated with significantly increased risk of recurrence (risk ratio estimate: 2.72, p=0.0089).

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Conclusions—Gain of 1q may provide a valuable prognostic marker to stratify therapy for patients with FHWT. A confirmatory study is necessary before this biomarker is incorporated into risk stratification schema of future therapeutic studies.

Keywords

Wilms tumor; prognosis; chromosome 1; kidney; pediatrics

Introduction

Wilms tumor is the most common primary renal tumor occurring in childhood, with approximately 500 cases identified each year in the United States.[1] The majority of Wilms tumors occur in children younger than five years of age, and are characterized by a triphasic histologic pattern consisting of blastemal, stromal, and epithelial elements. A small minority of Wilms tumors contain anaplastic histology consisting of nuclear enlargement, nuclear atypia, and irregular mitotic figures, which is associated with increased relapse risk [2–3]. The absence of anaplastic features identifies patients with favorable histology Wilms tumor (FHWT), which represents the patient population analyzed in this study.

Multidisciplinary collaboration and research through the National Wilms Tumor Study Group (NWTSG), now the Renal Tumor Committee of the Children's Oncology Group (COG), as well as the European pediatric cooperative groups has led to dramatic improvements in survival for the majority of patients with FHWT.[4–10] Multiple patient and tumor factors have been identified as prognostically significant in the NWTSG/COG patient cohorts, including tumor histology,[2] disease stage,[11] tumor-specific loss of heterozygosity (LOH) for chromosomes 1p and 16q,[10,12] and patient age and tumor weight.[13–15] While the majority of patients with FHWT have good outcomes, many patients still experience recurrence and death from disease, even among patients with lower disease stages. Also, present therapeutic approaches expose patients to significant risk for both immediate and late morbidity and mortality, including cardiac[16] and hepatic toxicity,[17] secondary malignancies,[18] and pregnancy complications.[19] Additional prognostic factors are necessary to prospectively identify those patients at the time of diagnosis who are at greater risk for recurrence. LOH of 1p and 16q in Wilms tumor was first described from a cohort of NWTSG patients enrolled on the third and fourth National Wilms tumor studies (NWTS-3 and -4) which demonstrated inferior outcomes for patients with LOH for 16q, and trends toward inferior outcome for patients with LOH for 1p.[12] Further work using prospectively gathered samples as part of the 5th National Wilms tumor study (NWTS-5) provided compelling data that patients with combined LOH for 1p and 16q had inferior event-free and overall survival, regardless of stage of disease. However, only 4.6% (76/1656) of FHWT patients had tumors with combined LOH 1p/16q, and combined LOH for 1p/16q was present in only 9.4% (20/213) of relapses.[10]

A number of additional studies have identified other genetic changes in FHWT that are associated with outcome. The change that has been consistently reported in all such studies is gain of chromosome 1q.[20–31] Despite the strength of the data previously reported, all these studies were performed in convenience samples that were not consistently treated.

Further, limited data have been published showing the relationship of 1q gain and outcome within different tumor stages. Importantly, 1q gain and LOH at 1p/16q are not independent events. LOH at 1p and 16q often arises through chromosomal translocations that also result in 1q gain. The goal of the current study was to determine if 1q gain, analyzed using multiplex ligation-dependent probe amplification (MLPA), is associated with event-free and overall survival in a carefully stratified cohort of FHWT patients enrolled on the National Wilms Tumor Study-4 (NWTS-4). The study also aimed to better define the relationship between 1q gain and 1p/16q LOH.

Methods

Clinical Samples

Patients included in this study were drawn from those who registered prospectively after providing informed consent on the Pediatric Oncology Group Study 9046, "A Molecular Genetic analysis of Wilms Tumor." While some of the PO6 9046 patients were enrolled on NWTS-3, the entire cohort from this study came from patients enrolled on NWTS-4, which represents a slightly different cohort from that reported previously.[12] Requirements for inclusion into the current study were the presence of unilateral renal disease, FHWT confirmed by central pathology review, and registration with full eligibility and follow-up on NWTS-4.

Multiplex Ligation-Dependent Probe Amplification (MLPA)

MLPA was performed as previously described using a synthetic probe mixture and minor modifications.[32] Gene-specific left and right probes were created using AlleleID 7.70 (Premier Biosoft International, Palo Alto, CA). Probes for 1q were selected to include the minimum region of gain in 1q22-1q23 previously reported [25,30]. All probe and primer target-specific sequences are provided with controls in Table 1. Universal forward and reverse sequences were attached to the left and right probes, respectively. The probe sizes were increased by using random sequences to obtain a specific amplicon size. The right probe was modified with an addition of 5′ phosphorylation. The probes were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). A probe master mix contained a final concentration of each left probe of 2.6 nM and of each right probe 2.6 nM. Approximately 250 ng of DNA in a volume of 5 μL was denatured at 98°C for 5 minutes. A mixture of 1.5 μL of MLPA buffer (1.5 M of KCl, 300 mM of Tris-HCl, pH 8.5, 1 mM of EDTA) and 1.5 μL of a probe set (6.9 pM for each hemiprobe) was added. The mixture was heated for 1 minute at 95°C followed by 16 to 18 hours at 60°C to allow the MLPA hemiprobes to hybridize. Next, 32 μL of ligase-65 mixture (dilution buffer containing 2.6 mM of MgCl2, 5 mM of Tris-HCl, pH 8.5, 0.013% nonionic detergents, 0.2 mM of nicotinamide adenine dinucleotide, and 1 U of ligase-65 enzyme) was added to each sample for ligation of hybridized hemiprobes during 15 to 20 minutes of incubation at 54°C, followed by 5 minutes of incubation at 98°C to inactivate the ligase. The amplification step was carried out in a 25 μl reaction volume using: 2.5 μl of 10xPCR Gold buffer, 4 μl of 25 mM MgCl$_2$, 0.25 μl of AmpliTaq Gold (5U/μl) (ABI), 0.5 μl of dNTP, 1 μl of 10 μM universal forward primer and 1 μl of 10 μM universal reverse primer. The universal forward primer has a 6-carboxyfluorescein (FAM) fluorophore attached to the 5′ end which is used

to detect the amplicon during peak height analysis. The qPCR cycling conditions were as follows: 37°C for 30 min; 95°C for 10 min; 60°C for 30 s; 95°C for 30 s and 67°C for 30 s (35 cycles); 72°C for 20 min. Analysis of the MLPA PCR products for each gene was carried out on an ABI 3100-Avant genetic analyzer (Applied Biosystems-ABI, Foster City, CA) in a mixture of 10 μL of deionized formamide (ABI), 1 μL of PCR product, and 0.5 μL of marker including a ROX-labeled internal size standard (ROX-500 Genescan, ABI) by using POP 4 polymer (ABI).

Analysis

After separation by capillary electrophoresis, peaks corresponding to each probe were identified by GeneMapper analysis (ABI). Samples in which the smallest peak was <100 relative fluorescent intensity units were not analyzed. The raw peak area for each probe in each sample was divided by the average raw peak area for all probes in that sample. This normalized peak area was then divided by the normalized peak area of the reference samples. Those control probes with a coefficient of variation (CV) >20% were removed from the analysis. Only those samples with at least three control probes remaining were scored. Test probes >1.25 were considered gained and those <0.75 were considered to be lost. These levels were chosen empirically using the distribution of copy levels of control probes. Gain or loss for a chromosomal region was scored if at least two markers were gained or lost, respectively. Scoring was performed without knowledge of outcome, and without knowledge of 1p and 16q LOH status.

Statistical Analysis

The two end points were eight-year event-free survival (EFS) and overall survival (OS). EFS and OS curves were estimated using the method of Kaplan and Meier[33] and compared using the log-rank test.[34] Relative risks were calculated using the Cox proportional hazards model.[35] Tests of correlation of 1q gain status and patient or disease characteristics were performed using the standard chi-square test for contingency tables.

Results

212 patients met the patient and sample criteria for this study; included were Stage I (82 patients, 39%), Stage II (54 patients, 25%), Stage III (46 patients, 22%), and Stage IV (30 patients, 14%). This distribution is comparable to that seen overall in the NWTSG studies.[9] The eight-year EFS estimate was 87% (95% CI 82%, 91%) for the entire cohort of 212 patients. Tumors of 58/212 patients (27%) displayed evidence of gain of 1q using the methods and criteria described. The eight-year EFS was 76% (95% CI 63%, 85%) for those with 1q gain and 93% (95% CI 87%, 96%) for those who lacked 1q gain (p=0.0024, Figure 1). The OS was 89% (95% CI 78%, 95%) for those with 1q gain, and 98% (95% CI 94%, 99%) for those who lacked 1q gain (p=0.0075, Figure 2). There were too few EFS events (27 total) to analyze the effect of 1q gain within stage subsets. However, there was no indication that 1q gain correlated with disease stage, as gain of 1q was identified in 20%, 31%, 37%, and 27% of patients with overall Stages I, II, III, and IV, respectively (p=0.16). Estimating the effect of 1q gain on EFS after stratification for stage, 1q gain was associated with a significant increase in the risk of recurrence (risk ratio estimate: 2.72, p=0.0089). For

OS, tumors with 1q gain trended strongly toward an increased risk of death (risk ratio estimate: 3.08). However, this did not reach statistical significance (p=0.067), possibly due to the small number of deaths overall (9 deaths total in cohort).

Similarly, the association with outcome was determined for 1p and 16q copy number loss. Retention of both 1p and 16q (no loss at either allele) was identified in 142 patients (estimated EFS 89%, 95% CI: 83%, 93%), isolated 16q loss was identified in 25 patients (estimated EFS 84%, 95% CI: 63%, 94%), isolated 1p loss was identified in 14 patients (estimated EFS 79%, 95% CI: 47%, 93%), and 7 patients had combined 1p and 16q copy number loss (estimated EFS 71%, p=0.29, 95% CI: 26%, 92%). There were too few cases with combined 1p and 16q loss to reliably assess whether this subset was associated with poorer EFS. It is notable that in this study tumors with 1q gain were more likely to have 16q loss (42.6%) or 1p loss (32.5%) than tumors without 1q gain (7.9% and 5.4% respectively, p<0.0001 for both comparisons).

Copy number loss is not always equivalent to LOH, since LOH may not result in a net loss of genetic material. LOH resulting from somatic recombination is copy number neutral, as is LOH resulting from chromosome loss and reduplication of the remaining homologous chromosome. LOH data for 1p and/or 16q (performed using microsatellite analysis and reported previously) were available for many of the tumors analyzed in this study. LOH data were available for 27% and 98% of the samples tested in this study for 1p and 16q copy number, respectively, and the concordance was 95% and 97%, respectively. The discordance was largely due to the identification of copy neutral LOH in 2/7 cases with 1p LOH and 5/35 tumors with 16q LOH. Approximately two-thirds of tumors with 1q gain contained translocations between chromosomes 1 and another partner chromosome (Figure 3A). While a number of different chromosomal partners were seen in the cohort, the most common translocation partner was chromosome 16. The majority of the remaining patients with 1q gain contained the isochromosome 1q, i(1q), which results in gain of 1q and loss of 1p copy number (Figure 3B). Similarly, approximately 40% of patients with 16q loss demonstrate t(1q;16q), all of which retained der(16) and lost der(1) (Figure 3B). Many also contained duplication of the remaining intact chromosome 1, resulting in monosomy 16q, trisomy 1q, and two identical copies of 1p. This results in LOH for 1p (copy neutral) and 16q. Of note, in one-third of tumors, 16q loss was a result of unbalanced translocations with other partner chromosomes. Such translocations involved a mixture of duplications and losses similar to that seen in t(1;16). Twenty-one per cent demonstrated loss of the entire chromosome 16.

Discussion

The central goal of the Children's Oncology Group Renal Tumor Committee is to increase survival of patients with FHWT and minimize toxicities associated with therapy. Although the clinical features of patient age and tumor stage are valuable predictors of relapse risk in patients with FHWT, they have limited sensitivity and specificity for recurrence. Novel biomarkers are needed to enhance the current risk stratification schema, thereby allowing augmentation of therapy for patients with high risk of recurrence and reduction of therapy for patients with low risk of recurrence. This study sought to evaluate 1q gain as a possible biomarker for unfavorable prognosis in patients with FHWT.

The association of chromosomal changes with relapse in patients with FHWT has been suggested by a number of publications, all of which show similar findings. In the largest study, 127/195 tumors that subsequently relapsed had abnormal karyotypes.[20] The most common changes overall were gain of chromosomes 1q (28%), 8 (24%), and 12 (38%), and loss of 1p (13%), 11q (9%), 16q (19%), and 22 (10%). A stepwise Cox proportional hazards regression demonstrated the significant independent predictors of risk to be 1q gain (relative risk (RR) 3.4, p=0.005), stage IV disease (RR 5.0, p<0.001) and monosomy 22 (RR 5.9, p<0.001). Copy number changes of 1p, 1q, and 16q were often inter-related due to recurrent unbalanced chromosomal abnormalities. This group reported similar rates of unbalanced translocation with chromosome 16 and i1q formation as was seen in the current study.

The NWTSG focused on LOH as a marker for relapse. Analysis of 232 children registered on NWTS-3 and NWTS-4 whose tumors were FHWT or WT with anaplasia demonstrated LOH for markers on 1p and 16q in 12% and 17% of patients, respectively, and each was associated with a poorer RFS and OS when adjusted for stage.[12] This association was prospectively confirmed in 1727 informative FHWT registered on NWTS-5.[10] A relative risk of relapse of 1.56 and 1.49 was associated with 1p and 16q LOH, respectively, stratified by stage, with similar RR for OS seen. When LOH for 1p and 16q were combined, among the 970 patients with low-stage disease the RR for death was 4.25; and among the 686 patients with advanced disease (stage III, IV) the RR for death was 2.66. On the basis of these studies, children registered on the current COG protocols are stratified according to their combined 1p and 16q LOH status. FHWT patients with combined LOH of 1p and 16q are treated on the current COG renal tumor protocols with intensified therapy with an aim to improve EFS and OS for these patients; those containing both 1p and 16q LOH are treated more aggressively for each stage. However, even if intensified therapy is shown to be beneficial for this group, this will not impact the majority of patients who will relapse, as 1p and 16q LOH is present in only 4.6% of FHWT, and is predictive of only 9.4% of all relapses. The relative risk of relapse associated with LOH of 1p alone or 16q alone was not strong enough to warrant the risks of intensifying therapy in the current COG renal protocols.

In more recent years, classic comparative genomic hybridization (CGH) confirmed the most frequent alterations in FHWT to be gain of 1q, 8, and 12, and loss of 1p, 11p, 16q, and 22.[25] CGH analysis found only gain of 1q to be significantly associated with adverse outcome. Gain of 1q was observed in 27/46 relapsed FHWT (59%) compared with 5/21 (24%) 1q gain in non-relapsed FHWT (p =0.019, RR 2.5). While most tumors with 1q gain showed gain of the entire long arm, six tumors demonstrated gain of smaller regions, with the smallest region of common gain spanning 1q21-25. In eight cases (25%), 1p loss coexisted with 1q gain; and, of 27 relapse cases with 1q gain, corresponding loss of either 1p or 16q was identified in 26% and 37% of cases, respectively. These observations support a strong association between 1q gain and poor outcome, and suggest that the unbalanced chromosomal abnormalities mentioned above result in 1q gain. These observations were confirmed by the analysis of 76 FHWT using BAC array CGH, which further narrowed the recurrent region of gain to 1q22-1q23.[25]

A major limitation of the above studies was that none was performed in a prospectively identified patient cohort consistently treated on a cooperative group protocol. In the current study, the cohort was derived from a prospective clinical and biologic trial, and analyzed 1q gain using MLPA, which is capable of measuring both gain and loss of potential biomarkers.[32] MLPA is robust, flexible, can be reliably multiplexed, and is inexpensive. It does not require a source for comparison germline DNA and can be performed on archival tissue. MLPA has become rapidly accepted in the research community, and has entered the clinical realm. This study included probes within the minimal region of gain 1q22-23 (Table 1), and was also able to include probes to 1p and 16q in order to correlate 1q gain with 1p and 16 loss.

This study reports that gain of 1q was associated with a 17% absolute reduction (p=0.0024) in eight-year RFS and a 9% absolute reduction (p=0.0075) in eight-year OS. Further, 1q gain was distributed relatively evenly among tumors of disease stages I-IV. While true within-stage analysis was not possible due to the small sample size for each disease stage, the prognostic significance of 1q gain remained (relapse RR 2.72, p=0.0089) when controlling for disease stage. The RR of death was slightly below usual significance levels (RR 3.07, p=0.067) but analysis was limited by the small number of deaths in the overall cohort (9 deaths of 212 subjects). Further, this study confirms a strong association between 1q gain and 1p and 16q loss. The important gene or genes on 1q that contribute to the reduction in survival remains an enigma.

In conclusion, these findings suggest that 1q gain may indeed be useful to stratify therapy in future therapeutic trials for FHWT. This will require validation in an independent set of tumors. This will be accomplished utilizing >1400 patients registered on NWTS-5. This larger group of patients will enable the evaluation of smaller subsets based not only within stage, but also on 1p and 16q copy number and LOH status. While the overall prognosis for FHWT is good compared to many other malignant diseases, there is significant variation among patients at higher stages of disease.[36] Roughly 15% of stage III and 25% of stage IV FHWT patients will experience relapse with current standard therapy.[10] Given the significant heterogeneity of patients especially within stage III and IV FHWT, the addition of 1q gain to the existing prognostic framework of clinical, pathologic, and biologic features has the potential to substantially improve the accuracy of risk stratification for appropriate therapy selection across all stages of FHWT. As a result, it may be possible to not only intensify therapy early for those patients at higher risk of relapse or death but also to decrease therapy to those patients with excellent RFS and OS who lack markers of high risk disease. Given this significantly higher percentage of patients with 1q gain compared with LOH 1p and/or 16q, 1q gain has the potential to lead to significant improvement in patient outcomes if similar results are seen in validation studies.

Acknowledgments

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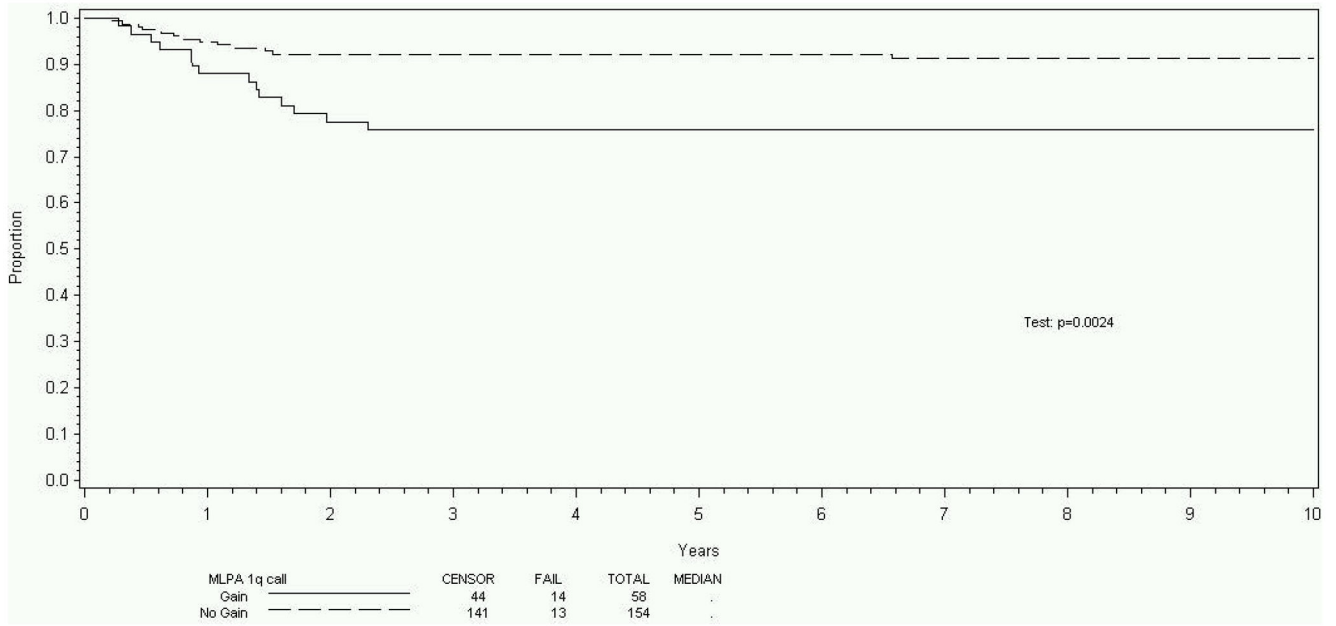
Figure 1.
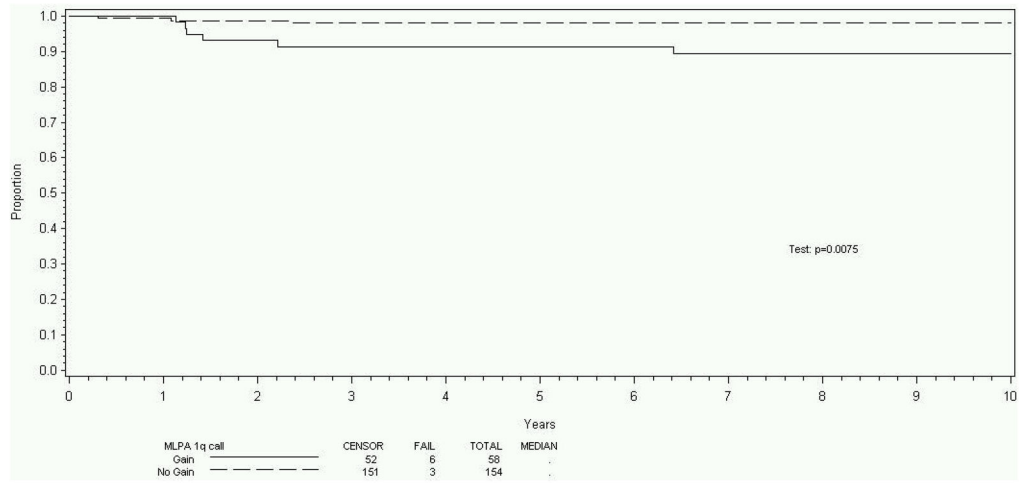Event-free survival stratified for 1q gain

Figure 2.
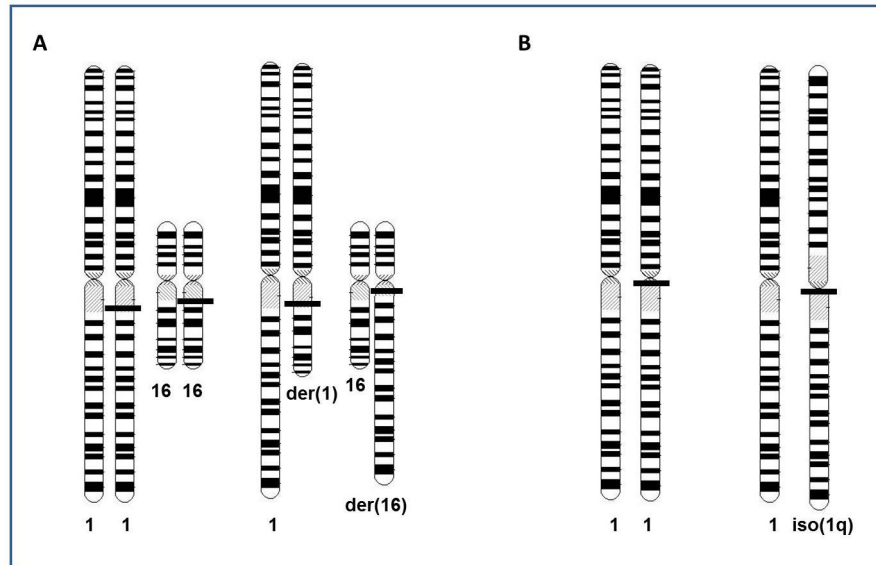Overall survival stratified for 1q gain

Figure 3.

Mechanisms responsible for 1q gain

A. Translocations involving chromosome 1 (the most common also involves chromosome 16). All tumors with t(1;16) examined thus far show loss of the der(1) and retention of the der(16). This results in loss of copy number and LOH for 1p and 16q. Tumors also variably show duplication of the normal chromosomes 1 and/or 16. While this increases the copy number for these chromosomal arms, because there are two exact copies, LOH is seen. B. Isochromosome 1q results from the development of a derivative chromosome 1 that contains two mirror-image copies of 1q. The normal chromosome 1 remains, resulting in 1q gain and 1p LOH. Again, the normal chromosome 1 is often duplicated, resulting in copy neutral LOH for 1p.

Table 1

Probe and primer target-specific sequences

Gene	Left Specific Sequence	Right Specific Sequence
LZIC (1p36)	TGGAGGTTTGTGCAATTTGAGACCGGTC	GGCACTGTGCAGAGATCAGAGTACTAAG
CAMTA1 (1p36)	CACTTGTTCATGGGCGCAGCA	AAGAAGAGGGATCCACAGAGCTGGA
AJAP1 (1p36)	CTTATTCCTGTGGCCTTCGTGTCTGAGA	AATGGTTTGAAATCTCCTGCTGACTGGC
DFFB (1p36)	TCGCGCCTTTGCTTTCCTGAG	CCTTCTGAGTAAGGTAATGTGGTGTCC
SETDB1 (1q21)	CGAGTTAACCGCAAGATGGGCTTTCATGTTATC	TATAAGACACCTTGTGGTCTCTGCCTTCGGACA
ADAM15 (1q22)	GAAAGAGGCTGGGACACCAACTCCTCCT	TGGAACTTTCACTTCCCGCTGCTGTCTT
SMG5 (1q22)	CCTGGCAGGCAGCAAGTACTA	TAATGTGGAAGCCATGTATTGCTACC
CACNA1E (1q25)	GCTGTGCGTGTCCTGCGGCCTT	TGAAGCTCGTGTCAGGGATACCTA
NFATC3 (16q22)	GAAGTGCAACCTAAAACTCATCATCGAG	CCCATTATGAAACTGAAGGTAGCCGAGG
KARS-2 (16q23)	GCATTGATCGAGTCGCCATGTTTCTCAC	GGACTCCAACAACATCAAGGTACGTAGC
CDH15 (16q24)	GACGCCTACGACATCAGCCAGCTG	CGTCACCCGACAGCGCTGAGCCT
TUBB3 (16q24)	GACCGGACGGTGAGTCAGCCTTAAG	CCCGGCACCAGACCCCTCTGAGGAT
FABP1 (2p11)*	CAGTGGACAGTCTGGTCGGCA	GAGCCGCAGGTCAGTCGTGAAGAGG
MITF (3p14)*	GTGCGGAAAATTCCATTTGGTGTTCGCC	GGCTGATGTGCAAGTAAAAGCAGGGAAT
TSC1 (9q34)*	GCAAGTGCAAAGGCCTTGAGCAAGAAAGAACCA	GTATTCCTGTGTTTGGGAAGACTGGGACTAGAGC
RET (10q11)*	GGACAGGCTAGCTAGCTGTGTTAGAAGTAGCAA	TGACAATGACCAAGGACTGCTACACCTCTGATT
DIABLO1 (12q34)*	CAGAGCAGACAGAACCGCGGA	GCTTCAGGGTGGAAGATTCGTGGAA
FMR1 (X)*	CATTACAGAATACCTCCAGTGAAGGTAGTCGGCT	GCGCACGGGTAAAGATCGTAACCAGAAGAAAGA