Recent cytogenetic and molecular investigations have improved our understanding of endometrial stromal tumors, including sarcomas (ESS), and helped redefine their classification into more pathogenetically meaningful categories. Because much more can be gained through such studies, we add information on another 22 ESS examined by karyotyping, PCR analysis, expression array analysis, and transcriptome sequencing. In spite of the known preference for certain pathogenetic pathways, we found considerable genetic heterogeneity in high-grade (HG) as well as in low-grade (LG) ESS. Not all HG tumors showed a YWHAE-NUTM1 chimeric transcript and as many as six LG ESS showed no hitherto known ESS-related fusions. Among the transcripts identified by transcriptome sequencing and verified by Sanger sequencing, new variants of ZC3H7-BCOR and its reciprocal BCOR-ZC3H7 were identified as was involvement of the CREBBP and MLLT4 genes (both well known leukemia-related genes) in two new fusions. FISH analysis identified a known EPC1-PHF1 fusion which led to the identification of a new variant at the molecular level. The fact that around 70 genes were found differentially expressed, by microarray analysis, when comparing LG ESS showing ESS-related fusions with LG ESS without such transcripts, underscores the biochemical importance of the observed genetic heterogeneity and hints that new subgroups/entities in LG ESS still remain undiscovered.
respectively (Micci et al., 2006; Panagopoulos et al., 2012; Micci et al., 2014). Fusions of the JAZF1, SUZ12, and PHF1 genes appear to be frequent, although certainly not ubiquitous, in LGESS, but have also been found in other types of EST (Chiang et al., 2011).

Lately, another two fusion transcripts have also been identified in LGESS. ZC3H7B-BCOR stems from an X:22-translocation whereas MBTD1-Cxorf67 is caused by a t(X;17) (Panagopoulos et al., 2013; Dewaele et al., 2014).

The cytogenetic literature contains altogether 19 cases with a t(10;17)(q22;p13), all of them belonging to the HGESS subtype with more aggressive clinical behavior. Lee et al. (2012a) described a YWHAE-NUTM (previously known as YWHAE-FAM22) chimeric fusion brought about by this rearrangement.

Gene expression profiles exist for 21 EST (Lee et al., 2012a; Davidson et al., 2013; Dewaele et al., 2014) of which six tumors were described as HGESS/UUS showing a 10;17-translocation (Lee et al., 2012a; Dewaele et al., 2014), six were LGESS characterized by a specific 7;17-translocation (Lee et al., 2012a; Dewaele et al., 2014), two cases were LGESS showing a t(X;17), and the remaining seven were LGESS with no karyotypic and/or genetic information (Davidson et al., 2013).

We wanted to increase the knowledge on ESS analyzing a series of 27 tumors (19 LGESS and eight HGESS) using different approaches that vary from standard techniques such as karyotyping and PCR to the more modern microarray and sequencing methodologies.

MATERIAL AND METHODS

Tumors

The material consisted of 27 samples from primary EST (Table 1) surgically removed at The Norwegian Radium Hospital. All tumors showed presence of endometrial differentiation. Eight of the tumors were diagnosed as HGESS whereas 18 were LGESS. HGESS are diagnosed based on morphology and the presence of diffuse (>70%) nuclear staining for cyclin D1 (WHO, 2014). Case 9 was an ESS showing areas with a mixture of LG and HG differentiation which changed from slide to slide. The case was described as not otherwise specified (NOS). The tumor biobank has been registered according to national legislation and the study has been approved by the Regional Committee for Medical Research Ethics South-East, REK; project numbers S-07194a and 2.2007.425.

G-banding and Karyotyping

The specimens intended for cytogenetic analysis (n = 18) were mechanically and enzymatically disaggregated and short-term cultured as previously reported (Micci et al., 1999). The subsequent cytogenetic analysis and karyotypic description followed the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN, 2009).

Molecular Investigations

RNA extraction

RNA was extracted from the tumors using TriZol reagent (Life Technologies) with a homogenizer (Omni THQ Digital Tissue Homogenizer, Kennesaw, GA). The RNA quality was evaluated using the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA). cDNA was synthesized using the iScript kit and random primers (Bio-Rad Laboratories). All procedures were done according to the manufacturers’ recommendations.

Polymerase chain reaction (PCR)

Reverse transcriptase (RT) PCR was used to investigate the presence of known fusion transcripts in all the samples as well as to validate the results from RNA sequencing. The primers used are listed in Table 2. The conditions used for PCR reactions were as previously reported (Micci et al., 2006; Panagopoulos et al., 2012, 2013; Dewaele et al., 2014). All PCR products obtained were sent for direct sequencing (Sanger Sequencing) at GATC Biotech (http://www.gate-biotech.com/en/sanger-services/lightrun-sequencing.html).

Microarray

Twenty-four samples were analyzed for gene expression. The microarray experiments were performed at the Norwegian Genomics Consortium in Oslo (http://oslo.genomics.no/) using the Illumina iScan which is based upon fluorescence detection of biotin-labeled cRNA. The experiments were performed as previously described (Micci et al., 2013). Bead summary data was imported into GenomeStudio to remove control probes and to produce a text file containing the signal and detection P values per probe for all
samples, as well as inputing missing data using the k-nearest neighbor algorithm (Tech note: Inputing HumanHT-12 Expression BeadChip Data; Illumina). The text file was imported into J-Express Pro 2011, and signal intensity values were quantile normalized (Bolstad et al., 2003) and log transformed (base 2). Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) was used to look for differentially expressed genes. To obtain manageable datasets, differentially expressed genes were defined by a fold change >2 and q value ≤ 0.05.

RNA sequencing

A total of 3 µg of RNA from 18 ESS with available RNA was sent for high-throughput paired-end RNA-sequencing at the Norwegian Sequencing Centre, Ullevål Hospital (http://www.sequencing).

| Case | ESS subtype | RNA-seq | Microarray | Karyotype | Fusion transcript |
|------|-------------|---------|------------|-----------|------------------|
| 1    | HG          | yes     | yes        | 60~70,add(1)(q32),del(12;22)(p13;q13),add(19)(p13), +1~2,inc[p9]/46,XX[10] | YWHAE-NUTM ILF3-CATSPERD RarbAPG11-RBKS |
| 2    | HG          | yes     | yes        | 53~71 < 3n>,XXX,+(1)(q10),+2,+2~3,del(2;2),del(9)(q13),-11,-12,-13,-14,-15,-16,-17,-17,-19,+20, +add(20)(p13), +21, +21, +11mar[cp14] | GPR180-DZIP1 WIPF2-LASP1 |
| 3    | HG          | yes     | yes        | 118~125,del(1)(q11)x3,i(1)(q10)x2,add(2)(p13~15),i(17)(q10),inc[cp5] | RNF126-NDUFA11 RPH3AL-BZW2 |
| 4    | HG          | yes     | nd         |           | YWHAE-NUTM       |
| 5    | HG          | yes     | nd         |           | YWHAE-NUTM       |
| 6    | HG          | yes     | nd         |           | YWHAE-NUTM       |
| 7    | HG          | yes     | nd         |           | MLL4-RB1         |
| 8    | HG          | yes     |            | 46,XX,(10;17)(q22;p13)[17]/46,XX[1] | EPC1-PHF1 |
| 9    | NOS         |        |            | 46,XX,del(X)(p21)[2]/77~80,XX,X,+3,-6,+del(7)(p11),+8,+11, -12,+16,+18,+19,+20,+20,+21,+21[cp2]/46,XX[11] | |
| 10   | LG          | yes     | yes        | 45,XX,-10[16]/46,XX[1] | |
| 11   | LG          | yes     | yes        | 46,XX,(7;17)(p15;p21)[10]/46,XX,del[10],+13,-15[3] | JAZFI-SUZ12 |
| 12   | LG          | yes     | yes        | 82~90,XXXX,t(6;7)(q16;p15)x2,del(1)(q11)x2,add(1)(p15)x2, -add(12)(q13),del(20)(q13),-22,-22,+mar[cp9] | JAZFI-SUZ12 |
| 13   | LG          | yes     | yes        | 46,XX,del(X;22)(p11;13),del(3;4)(p23;q27)[8]/46,XX[8] | ZC3H7B-COR |
| 14   | LG          | yes     | yes        | 46,XX,(2;14)(q21;p24),-3,add(3)(q21~24),der(5)(3;5)(q13~12),p15, +del(5)(q13),der(5)(5;6)(q13;q21),del(9)(p13),-16,der(17)(12;17) | GMP5-SYNPR |
| 15   | LG          | yes     | yes        | 47~51,XX,+(1)(q10),+2,+11,+13,mar[cp9]/46,XX[6] | KDM2B-CREBBP |
| 16   | LG          | yes     | yes        | 46,XX,(1;6)(p32~34;p21)[15] | MEAF6-PHF1 |
| 17   | LG          | yes     | yes        | 42,X,del(X;22)(p1;1p1;11),der(1;9)(q25;22~24)ins(1;1)(q25;1), der(9)(1;9)(q25;22),-10, der(13;15)(q10;q10),-21,der(22)(X;22) (p11;q13),+r[20] | ZC3H7B-COR |
| 18   | LG          | yes     | nd         |           | PXDN-XIRP2       |
| 19   | LG          | yes     | yes        | nd         | HZAFF-FHMHB1     |
| 20   | LG          | yes     | nd         |           | WTIP-BIRC6       |
| 21   | LG          | yes     | nd         |           | ZC3H7B-COR*      |
| 22   | LG          | yes     | nd         |           | NAIP-OCLN        |
| 23   | LG          | yes     | nd         |           | JAZFI-PHF1       |
| 24   | LG          | yes     | nd         |           | JAZFI-SUZ12      |
| 25   | LG          | yes     | nd         |           | JAZFI-PHF1       |
| 26   | LG          | yes     | nd         |           | JAZFI-SUZ12      |
| 27   | LG          | yes     | nd         |           | EPC1-PHF1*       |

*Case already published: cases 13 and 17 in Panagopoulos et al., 2013; case 16 in Panagopoulos et al., 2012; case 22 in Micci et al., 2006; case 25 in Micci et al., 2014.

*bnd: not done.

*cNew variant of the fusion.
The sequencing was performed using the Illumina HiSeq 2000 instrument. The Illumina software pipeline was used to process image data into raw sequencing data and only sequence reads marked as "passed filtering" were used in the downstream data analysis. The FASTQC software was used for quality control of the raw sequence data (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). An average number of 93 million sequence reads (range 32 to 180 millions) was obtained from transcriptome sequencing. We used the fusion discovery software FusionMap (release date 2012-04-16) (Ge et al., 2011) and the pre-built Human B37 and RefGene from the FusionMap website (http://www.omicsoft.com/fusionmap/) as well as the Fusion Catcher software (version 0.99.3a beta-April 15, 2014) with the associated ENSEMBL, UCSC, and RefSeq databases automatically downloaded by FusionCatcher (https://code.google.com/p/fusioncatcher/) (Kangaspeska et al., 2012) as an additional tool to detect fusion transcripts.

RESULTS

Karyotyping

The 18 samples which were cytogenetically analyzed all showed an abnormal karyotype (Table 1). Thirteen of them have not been reported before whereas five karyotypes have been published by our group (cases 13, 16, 17, 22, and 25) (Micci et al., 2006; Panagopoulos et al., 2012; Panagopoulos et al., 2013; Micci et al., 2014). Among the 13 new cases, four were HGESS, eight were LGESS, and one (case 9) was an ESS NOS. Only one HGESS (case 8) showed a 10;17-translocation as the sole karyotypic abnormality. Cases 1 and 2 presented triploid karyotypes showing both structural and numerical aberrations, whereas case 3 had a near-pentaploid karyotype with multiple structural rearrangements only few of which could be identified. None of the aberrations of these three HGESS showed involvement of any of the chromosomes, let alone chromosome bands, known to be involved in EST-specific rearrangements. Among the eight LGESS, three tumors (cases 11, 12, and 26) showed the specific 7;17-translocation either as the sole karyotypic abnormality. Cases 1 and 2 presented triploid karyotypes showing both structural and numerical aberrations, whereas case 3 had a near-pentaploid karyotype with multiple structural rearrangements only few of which could be identified. None of the aberrations of these three HGESS showed involvement of any of the chromosomes, let alone chromosome bands, known to be involved in EST-specific rearrangements. Among the eight LGESS, three tumors (cases 11, 12, and 26) showed the specific 7;17-translocation either as the sole karyotypic aberration (case 11) or among other abnormalities (cases 12 and 26). Case 27 showed a 6;10- and a 10;6-insertion. Case 10 had monosomy 10 as the sole aberration, whereas in the remaining three LGESS (cases 14, 15, and 24), a near-diploid karyotype was found showing both numerical and structural aberrations. Case 9 had two unrelated clones, one with a possible del(X)(p21), the other, triploid, with both structural and numerical aberrations.
RT-PCR

All tumors were tested for the fusions known to be associated with ESS. A summary of the results is shown in Table 1. The fusions were all confirmed by Sanger sequencing. PCR investigations confirmed the presence of all fusions previously published (Micci et al., 2006; Panagopoulos et al., 2012, 2013; Micci et al., 2014). The YWHAE-NUTM fusion was found in five cases, all of them HGESS. The JAZF1-SUZ12 fusion transcript was identified in four LGESS. A JAZF1-PHF1 fusion was identified in one tumor (case 24). Case 9, the ESS NOS, showed a specific fusion between the EPC1 gene, mapping on 10p11, and the PHF1 gene, from 6p21. Fusion of the same genes was also found in case 27 but with a new variant, i.e., the junction involved exon 9 of the EPC1 (accession number NM_025209.3) (Fig. 1).

RT-PCR was used to validate those fusions obtained by sequencing analysis that showed higher seed count. An overview of the cases analyzed and the transcripts identified is provided in Table 1. In case 20, transcriptome sequencing (see below) identified the presence of a ZC3H7B-BCOR and its reciprocal BCOR-ZC3H7B fusion, previously described by Panagopoulos et al. (2013) as brought about by a X;22-translocation. Sequencing of the amplified cDNA fragments showed two transcripts from the ZC3H7B-BCOR fusion, one with involvement of exon 10 of ZC3H7B (accession number NM_017590.5) and exon 9 of BCOR (accession number NM_017745.5), the other...
showing a fusion between exon 12 of ZC3H7B and exon 9 of BCOR (Fig. 1). The reciprocal BCOR-ZC3H7B fusion was between exon 7 of BCOR and exon 13 of ZC3H7B.

Microarray

The original microarray data can be found in the public database Gene Expression Omnibus (GEO; available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73361). The microarray analysis of 24 ESS was performed taking into consideration the pathological subclassification of the tumors so that the expression profiles of the HGEss (7 tumors) were compared to those of the LGESS (17 tumors). The ESS classified as NOS was grouped with the LG tumors since it showed an EPC1-PHF1. We identified 514 differentially expressed unique genes in the comparison between the two groups, of which 187 genes were down-regulated in the HG compared to the LG-group of ESS and 327 genes were upregulated in the HG compared to the LG-group of ESS (Supporting Information Table S1). The data were plotted using Correspondence Analysis (CA) (Fellenberg et al., 2001) to look for the greatest covariance between samples and genes so as to see if any array behaved differently compared to the others. The CA-plot of the samples from the two groups (HG vs. LG) showed a distinct separation. Among the LGESS, several CA-plots were also performed to compare different subgroups based on the karyotypic aberration and/or specific gene fusion present. In these analyses, False discovery rate (FDR) <0.05 was used as cutoff. The comparison between tumors showing t(7;17) and the LGESS samples without specific chromosomal rearrangement and/or gene fusion (referred to from now on as OTHER) revealed around 70 differentially expressed genes (Fig. 2), all other comparisons showed less than ten. The comparison between tumors showing a t(X;22) and OTHER identified eight differentially expressed genes. Furthermore, the comparisons between the group of ESS with 6p-rearrangements versus t(X;22) or OTHER revealed no differentially expressed gene. A list of the genes and probes differentially expressed in each comparison can be found in Supporting Information.

RNA Sequencing

The Fusion Map and Fusion Catcher programs were used to find fusion transcripts in the samples (Liu et al., 2016). We focused only on the common transcripts. Five out of the 18 ESS sequenced showed a previously described fusion: cases 13 and 17 had a BCOR-ZC3H7B (Panagopoulos et al.,...
2013), cases 16 and 25 showed a MEAF6-PHF1 (Panagopoulos et al., 2012; Micci et al., 2014), and in case 20 the ZC3H7B-BCOR and its reciprocal BCOR-ZC3H7B transcript were found. Validation of the fusions by PCR using specific primer combinations revealed the presence of three transcripts in the latter case (see Results for PCR analyses). Because most of the putative fusions detected by both programs/algorithms may be false positives, we selected 22 different fusions with the highest seed counts from 13 tumors to validate their actual presence by PCR using specific primer combinations. PCR reactions for seven transcripts did not show any amplification of the putative fusion, but the remaining 15 transcripts could be amplified by PCR and direct sequencing (Supporting Information Table S2). For all but two transcripts (H2AFY-HMHB1 from case 19 and NAIP-OCLN from case 21), the two genes were found to be in-frame. The transcriptome sequencing did not detect any YWHAE-NUTM fusion in the seven HGESS with NGS data.

**DISCUSSION**

Recent cytogenetic and molecular investigations have improved our understanding of EST and helped classify them into more meaningful categories. These tumors typically demonstrate relatively simple karyotypes with specific chromosomal rearrangements (the majority of ESN, LGESS, and YWHAE-rearranged HGESS) or harbor complex cytogenetic aberrations lacking specific rearrangements (as in EUS/UUS). The 2014 WHO classification of EST (2014) incorporates molecular findings, and yet molecular testing is not routinely performed when these diagnoses are suspected. The molecular signature may be diagnostically helpful when dealing with cases of unusual location or morphology; furthermore, a better genetic characterization of these tumors may also provide prognostically and/or therapeutically relevant information. Because the cytogenetic and molecular literature on EST is still severely limited (Table 3), we add to the body of knowledge information on 27 ESS investigated using cytogenetic, PCR, expression array, and transcriptome sequencing analyses.

The karyotypic data on 13 of the ESS described here (Table 1) have not been reported before. Karyotypic complexity was observed in both HG and LG tumors. In five cases, a neat correspondence between karyotypic aberrations and molecular findings was identified, i.e., case 8 showed a 10;17-translocation leading to a YWHAE-NUTM fusion, cases 11, 12, and 23 had a specific t(7;17) and a JAZF1-SUZ12 fusion, and case 27 showed two insertions, an ins(6;10) and an ins(10;6), leading to an EPC1-PHF1 fusion. In case 9, on the other hand, a specific fusion was seen between the EPC1 gene, mapping on 10p11, and the PHF1 gene from 6p21, in spite of the fact that no rearrangement of these two chromosome bands was seen cytogenetically. Likewise, a JAZF1-PHF1 fusion was identified in one tumor (case 24) which in its karyotype had no visible rearrangement of 7p15 and/or 6p21. Evidently, cases of ESS exist that by karyotyping are “false negatives,” i.e., the pathogenetically crucial gene-level rearrangement takes place without visible change of the chromosomal morphology. The fact that some ESS may carry cryptic rearrangements leading to one of the known fusions characteristic of this tumor type has also previously been alluded to (Micci et al., 2014). Specific gene fusions may also possibly be hidden in rings and/or marker chromosome(s) that are part of incompletely described karyotypes.

The karyotypic data gave the impression of increasing complexity from LG to HG ESS, something that has also been seen by others. Flicker et al. (2015) performed aCGH on 30 EST showing an increasing number of copy number changes from ESN to LGESS and UES. In their study as well as in the present one, the chromosomal aberrations differed considerably among the groups, indicating that a linear tumor progression from one group to the next does not take place. Instead, classification into different entities based on genetic aberration pattern is more appropriate.

The microarray analysis identified 514 differentially expressed genes between HG and LGESS. Seventy-six of these genes are already known to be associated with cancer. No hint as to which of them may be the most important in ESS is at hand. The comparison between LGESS having a t(7;17) and/or JAZF1-SUZ12 fusion versus those showing 6p-rearrangements and/or PHF1 involvement showed no differentially expressed genes. In contrast, the comparison between tumors with t(7;17) and those with t(X;22) showed nine genes that were differentially expressed. These results show once more that despite the presence of different fusions, these tumors are quite similar and belong to the same subgroup as far as phenotype and gene expression pattern are concerned. The JAZF1-SUZ12, JAZF1-PHF1, EPC1-PHF1, MEAF6-PHF1, ZC3H7B-BCOR, and possibly also MBTD1-Cxorf67 gene fusions (admittedly, the
### Case Karyotype Reported by

#### 7;17-rearrangements

| Case | Karyotype | Reported by |
|------|-----------|-------------|
| 1    | 46,XX,t(7;13)(p15;q14),t(7;17)(p15;q11),del(11)(q21q23) | Fletcher et al., 1991 |
| 2    | 46,XX,t(7;17)(p15;q11),t(7;17)(p21;q12),del(11)(q32q21) | Sreekantaiah et al., 1991 |
| 3    | 46,XX,t(7;17)(p15-21;q12-21)/46,ident.-,+der(?)(t(?;t(?)) (t?q11)/45, Ident.-,dic(15;22)(p11;p11),+der(t)?(t) | Dal Cin et al., 1992 |
| 4    | 46,XX,del(6)(q15),der(6)(t;6;11)(p21;q11),add(7)(p21), t(?;7)(p15-21;q12-21),+9-11 | Pauwels et al., 1996 |
| 5    | 46,XX,der(7)(t;7;16)(p14-15;q22)t(7;9)(q22;q22),t(7;17) (p14-21;q11-21),der (?;7)(q7;9)(q22;q22),del(16)(q22)/ 47,ident,del(3)p13p23, +mar | Hennig et al., 1997 |
| 6    | 42-44,X,X,X,der(2)t(2;7)(p23;p15)t(2;15)(q35;q15),add(4) (p16),del(7)(p15q15),der(7)(7;17)(p4q12),add(8)(q24), -10,del(11)(p11)x(11-13),p15;14;4),del(15)(q15)-16, del(17)(q12),der(18)(16;18)(p11p11),add(19)(p13),-20, add(21)(q22),-2+2mar/78-83,identm2 | Iliszko et al., 1998 |
| 7    | 53-55,X,X,X,del(1)(p32),del(1)(p21),1 der(1)t(1;3)(p32;p21),1 del(3)(p21),1 der (3;15)(q10;q10),-5,1 der(6)add(6) (p11)add(6)(q27),add(7)(p11),+add(7) (p21),1+8-11-13, der(13;21)(q10q10),add(14)(p11),der(15)(t;6;15) (p21;p12), der(17)(t;3;17)(p21;p13)x2, +18,18add(19)(q13),-20, der(21;21)(q10q10),+2mar,dmin | Gil-Benso et al., 1999 |
| 8    | 46,XX,t(7;13)(p15;p13),t(7;17)(p15;q21) | Koontz et al., 2001 |
| 9    | 46,XX,t(7;17)(p15;q21) | Koontz et al., 2001 |
| 10   | 46,XX,t(7;17)(p15;q21) | Koontz et al., 2001 |
| 11   | 45,XX,del(7)t(7;16)(p14-15;q22)t(7;9)(q22;q22),t(7;17) (p14-21;q11-21),der (?;7)(q7;9)(q22;q22),del(16)(q22)/ 47,ident,del(3)p13p23, +mar | Hennig et al., 1997 |
| 12   | 46,XX,der(6)ins(6;7)(p21;q34q11)del(6)(p21),der(7)del(7) (p15;7)(p21;7q11),dup(7)(p22p15) | Micci et al., 2003 |
| 13   | 46,XX,inv(2)(p21q37),der(6)(6;6)(p21q6;7q21),t(7;6;7)(p21;7p15),del(6)q21) | Micci et al., 2003 |
| 14   | 47,X,der(10)(X)(X;16),1 +add(2)(q21),ins(2;22)(q31;c1q13), der(3;3;3;3;4;24q;2q22q22;q22),del(6)(p11),1 der(6)(3);6, der(7)(7;6;7)(p15;6;15)x(3;15)t(3;15);c(13;15) t(6;15)(X;6)(X;6)(c1q13),der(14)(1q14 psychedoq25;q32 | Regauer et al., 2008 |

#### 6p21-rearrangements

| Case | Karyotype | Reported by |
|------|-----------|-------------|
| 1    | 46,XX,del(5)(q23),der(5)(6;7)(p21;p21) | Laxman et al., 1993 |
| 2    | 46,XX,der(3)(3;7)(p12p12),der(6)(6;6)(p12;p21)t(6;7) | Hrynchak et al., 1994 |
| 3    | 38,XX,del(1)(q11)2,add(2)(p13),-add(4;4)(q14;4q14) (q35q11)add(4)(p12),add(6)(2),add(7)(7),add(7)(7),p11p13), -8,9,add(9)(q34),-10,add(10)(q24),-11,11,ins(12)(?q13), -14,14,15,ins(15)(q22),add(16)(q22),add(17)(q11), -18,18add(17)(q11),add(20)(p13),add(21)(p11), -22,add(22) (p11),+6mar | Sonobe et al., 1999 |
| 4    | 46,XX,der(6)ins(6;7)(p21q34q11)del(6)(p21),der(7)del(7) (p15;7)(p21q11),dup(7)(p22p15) | Micci et al., 2003 |
| 5    | 46,XX,inv(2)(p21q37),der(6)(6;6)(p21q6;7q21),t(7;6;7)(p21;7p15),del(6)q21) | Micci et al., 2003 |
| 6    | 47,X,der(10)(X)(X;16),1 +add(2)(q21),ins(2;22)(q31;c1q13), der(3;3;3;3;4;24q;2q22q22;q22),del(6)(p11),1 der(6)(3);6, der(7)(7;6;7)(p15;6;15)x(3;15)t(3;15);c(13;15) t(6;15)(X;6)(X;6)(c1q13),der(14)(1q14 psychedoq25;q32 | Micci et al., 2006 |

#### X;22-rearrangements

| Case | Karyotype | Reported by |
|------|-----------|-------------|
| 1    | 46,X,t(X;22)(p11q13),3(3;4)(p23q27) | Panagopoulos et al., 2013 |
| 2    | 42,X,del(22)(p11p11),der(1;9)(q25p22-24q11;1) (q25q7), der(9)(q19),10,der(13;15)(q10q10),11,der(22) t(X;22)(p11q13),+r | Panagopoulos et al., 2013 |

#### X;17-rearrangements

| Case | Karyotype | Reported by |
|------|-----------|-------------|
| 1    | 46,X,t(X;17)(p11q21)45,ident,del(4;22)(p15q13) | Amant et al., 2003 |
| 2    | 46,X,t(X;17)(p11q21)45,ident,del(4;22)(p15q13) | Dewaele et al., 2014 |
JAZF1 is a transcriptional repressor and SUZ12, PHF1, and MBTD1 are members of the polycomb group protein family involved in transcriptional repression. ZC3H7B is involved in protein-nucleic acid interactions, while BCOR is known to interact with polycomb group proteins; hence, ZC3H7B-BCOR probably mediates its oncogenic effects through aberrant epigenetic regulation (Panagopoulos et al., 2013). EPC1 is part of the nucleosome acetyltransferase of histone H4 complex.

**Table 3. (Continued)**

| Case | Karyotype Reported by |
|------|-----------------------|
| 1    | 45,XX,-10,der(19)(ins10;19)(p11;p13q13)/45,iddem.del(11)(p21-22)/45,iddem.del(1)(p21p35) Dal Cin et al., 1988 |
| 2    | 46,XX.del(7)(p22q32),del(12)(q14q22) Havel et al., 1989 |
| 3    | 76-18,XXXXX,del(3)(p12p21),del(12)(q13q22),inc Fletcher et al., 1991 |
| 4    | 49,XX,+7,+8, +9,del(14)(q11q22)(p13q12)/49,XX,+3,+7,+8,der(14) Laxman et al., 1993 |
| 5    | 80,XXI,del(1)(p11),if(1)(p10),del(6)(q24),del(5)(p11),del(6)(q12),del(12)(p11)add(16)(q12),add(19)(q13),inc Laxman et al., 1993 |
| 6    | 45-48,XX,del(3)(36q1q22)(p12p21),del(6)(q13q27), +i(19)(q10)/48,XX,del(3)(36q1q22)(p12p21),del(6)(q13q27), +i(19)(q10)/48,XX,del(3)(36q1q22)(p12p21),del(6)(q13q27), +i(19)(q10) Gunawan et al., 1998 |
| 7    | 48-50,XX,+2,+7/49-50,XX,+2,+7,+10 Iliosko et al., 1998 |
| 8    | 45-46,XX.del(6)(q21),del(12)(p13) Iliosko et al., 1998 |
| 9    | 46,XX,+t(13)(p13p25),i(8)(q10),dic(15;16)(p11q13) Iliosko et al., 1998 |
| 10   | 46,XX.del(6)(q22),add(20)(q13) Gil-Benso et al., 1999 |
| 11   | 46,XX.inv(5)(p13-14q23-31) Micci et al., 2014 |
| 12   | 45,XX,t(10;17)(q22;p13) Leunen et al., 2003 |
| 13   | 45,XX.add(3)(p11),der(9)(q7q9)(q11p24),del(10)(q11q26),t(10;17)(q22p13),der(11)(q31q11)p22q22)-13 Micci et al., 2003 |
| 14   | 46,XX,t(10;17)(q22p13),t(12;13)(q24q14) Regauer et al., 2008 |
| 15   | 47,XX,der(9)(del9)(p11)del9(q12),del(10)(q12p13),der(11) Amant et al., 2011 |
| 16   | 47,XX,der(9)(del9)(p11)del9(q12),del(10)(q12p13),der(11) Lee et al., 2012a |
| 17   | 46,XX,t(10;17)(q22p13) Lee et al., 2012a |
| 18   | 46,XX,t(10;17)(q22p13) Lee et al., 2012a |
| 19   | 46,XX,t(10;17)(q22p13) Lee et al., 2012a |
| 20   | 47,XX,t(10;17)(q22p13),t(11)(q12)p12q12),der(17)(q10p17)(q22p13),+19 Lee et al., 2012a |
| 21   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 22   | 46,XX,t(4;10;17)(q12;q22;p13) Lee et al., 2012b |
| 23   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 24   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 25   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 26   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 27   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 28   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 29   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 30   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 31   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 32   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |
| 33   | 46,XX,t(10;17)(q22p13) Lee et al., 2012b |

*Molecular analysis detected a MEAF6-PHF1 fusion.
whereas MEAF6 is part of histone acetyltransferase multi-subunit complexes. EPC1-PHF1 and MEAF6-PHF1 are believed to alter acetylation patterns of histone proteins resulting in unraveling of the heterochromatin and aberration gene expression (Avvakumov and Cote, 2007; Panagopoulos et al., 2012). All these fusions appear to combine genes that are known to be involved in transcriptional regulation, i.e., polycomb group complex-mediated and aberration methylation/acetylation; hence, their presumed oncogenic effects are probably mediated through altered transcriptional control in endometrial stromal progenitor cells.

In contrast, the comparison between 7;17-associated tumors and those described as OTHER (six tumors; cases 10, 14, 15, 18, 19, and 21), the ones showing no characteristic chromosomal rearrangement and/or fusion genes, revealed around 70 differently expressed genes (Supporting Information Table 3). Little cytogenetic information is available on these tumors as only three of them were sent for cell culturing and karyotyping. Case 10 showed monosomy 10 as the sole aberration in the karyotype, whereas case 14 showed as many as twelve chromosomal aberrations with both numerical and structural rearrangements, and case 15 had a hyperdiploid stemline with aberrations that could only be partly described in a composite karyotype. The cytogenetic literature contains information on seven ESS with no visible rearrangements of the chromosomal bands known to be involved in ESS-specific fusions, but no molecular information is available on these tumors allowing a classification based on molecular signature. We cannot but speculate that the said six cases of our series may represent a subgroup or variants of “classical” LGESS, despite their unequivocal histological classification.

Transcriptome sequencing was performed on the 18 tumors with available RNA. Surprisingly and alarmingly, none of the programs/algorithms used to screen the data for fusion transcripts, i.e., FusionMap and Fusion Catcher, detected the presence of YWHAE-NUTM in the four HGESS positive for this fusion by PCR. We did not check, however, if such a transcript was nevertheless present in the raw data and retrievable by use of the “grep” command (Panagopoulos et al., 2014a,b), but assume that to be the case. In spite of its immense investigative and attraction value, the next generation sequencing technology regrettably yields results that may be both falsely negative and positive (Panagopoulos et al., 2014a).

The only tumor showing a known ESS-related fusion transcript by NGS was case 20 in which a ZC3H7B-BCOR and its reciprocal BCOR-ZC3H7B were detected. However, PCR analyses confirmed the presence of variants of fusions compared to those previously reported by Panagopoulos et al. (2013). The three new transcripts fuse either exon 10 or exon 12 of ZC3H7B (accession number NM_017590.5) with exon 9 of BCOR (accession number NM_017745.5) in the two variants of the ZC3H7B-BCOR, whereas the variant of BCOR-ZC3H7B shows an in-frame fusion between exon 7 of BCOR and exon 13 of ZC3H7B (Fig. 1).

Twenty-two putative fusions were selected from those ranking highest based on the sequence analyses with both programs in the 13 cases examined. Amplification of cDNA with specific primers for each fusion confirmed the presence of the transcripts in 15 instances (Supporting Information Table 2). The seven transcripts that were not confirmed by PCR could be false positives/artefacts introduced during the RNA library preparation stage (Quail et al., 2008; Panagopoulos et al., 2014c) and/or the consequence of read-through transcription which occurs when the RNA polymerase continues beyond the normal termination sequence into an adjacent gene (Nacu et al., 2011). Among these 15 transcripts, GMPS-SYNPR (case 14) was particularly interesting since the two involved genes map to 3q25 and 3p14, respectively, and the karyotype showed two different rearrangements involving 3q. The correspondence between the molecular and karyotypic data adds credibility to the idea that this could be an important transcript in tumor development. Its function needs further investigation. The fusion is in-frame and involves exon 12 from the GMPS gene and exon 3 from SYNPR (Supporting Information Table 2). GMPS (accession number NM_003875) encodes a guanine monophosphate synthetase whereas SYNPR (accession number NM_144642) codes for a synatoporin. None of these genes has been reported to be directly involved in cancer; however, a study by Reddy et al. (2014) showed that GMPS is required for USP7-mediated stabilization of p53.

Case 15 showed another interesting fusion, between the lysine (K)-specific demethylase 2B (KDM2B) on 12q24 and the CREB binding protein (CREBBP) on 16p13. The in-frame fusion is between exon 22 of KDM2B (accession number NM_032590) and exon 2 of CREBBP (accession number NM_004380). The KDM2B gene (also known as Ndy1, FBXL10, and JHDM1B) encodes a...
member of the F-box protein family which is characterized by an ~40 amino acid motif (F-box). The F-box proteins constitute one of the four subunits of the ubiquitin ligase complex called SCFs (SKP1-cullin-F-box) which functions in phosphorylation-dependent ubiquitination. It has been found that KDM2B, an H3K36 histone demethylase implicated in bypassing cellular senescence and somatic cell reprogramming, is markedly overexpressed in human pancreatic ductal adenocarcinoma (PDAC), with levels increasing with disease grade and stage, and showing the highest expression in metastases (Tzatsos et al., 2013). KDM2B silencing abrogated tumorigenicity of PDAC cell lines exhibiting loss of epithelial differentiation, whereas KDM2B overexpression cooperated with KrasG12D to promote PDAC formation in mouse models. Gain- and loss-of-function experiments coupled to genome-wide gene expression and ChIP studies revealed that KDM2B drives tumorigenicity through two different transcriptional pathways. KDM2B repressed developmental genes through cobiinding with Polycomb group (PeG) proteins at transcriptional start sites, but also activated a module of metabolic genes, including mediators of protein synthesis and mitochondrial function, cobound by the MYC oncoprotein and the histone demethylase KDM5A. These results defined epigenetic programs through which KDM2B subverts cellular differentiation and drives the pathogenesis of an aggressive subset of PDAC (Tzatsos et al., 2013). The CREBBP gene is ubiquitously expressed and is involved in the transcriptional coactivation of many different transcription factors. First isolated as a nuclear protein that binds to cAMP-response element binding protein (CREB), the gene is now known to play critical roles in embryonic development, growth control, and homeostasis by coupling chromatin remodeling to transcription factor recognition. The protein encoded by this gene has intrinsic histone acetyltransferase activity but also acts as a scaffold to stabilize additional protein interactions with the transcription complex. It acetylates both histone and non-histone proteins. Chromosomal translocations targeting this gene have been associated with acute myeloid leukemia (AML) (Borrow et al., 1996; Panagopoulos et al., 2014b). The breakpoint identified in our case 15 is the same as that previously described (Panagopoulos et al., 2000) indicating that the fusion retains the oncogenic activities typical for AML (Borrow et al., 1996) also in this case of LGESS. This is the first time that CREBBP is found involved in a fusion transcript in solid tumors, and its importance in LGESS needs to be evaluated in larger series. The Mitelman Database for Chromosome Aberrations and Gene Fusions in Cancer (http://cgap.nci.nih.gov/Chromosomes/Mitelman) reports two ESS with alteration of chromosome 16 (Laxman et al., 1993; Iliszko et al., 1998) and one with 12q rearrangement (Havel et al., 1989), though with different breakpoint positions from those of the 6:12-translocation we identified. These rearrangements may possibly lead to a cryptic KDM2B-CREBBP fusion since the three tumors did not show any rearrangement typical of ESS.

The finding of similar, specific gene fusions in different tumor types is well known in cancer, and ESS-related fusions are no exception. Recently, ZC3H7B-BCOR, MEAF6-PHF1, and EPC1-PHF1 fusions have been found in ossifying fibromyxoid tumors (OFMT) (Antonescu et al., 2014) and a JAZF1-PHF1 was found in a cardiac ossifying sarcoma (Schoolmeester et al., 2013). Furthermore, a new PHF1-fusion variant, EP400-PHF1, was also described in OFMT (Gebre-Medhin et al., 2012; Endo et al., 2013). We do not have any explanation for such similarities between different mesenchymal tumors or the involvement of the CREBBP gene in leukemias and ESS; it seems that they are not tissue-specific although their tumorigenic role is unquestionable. The same can be said for the MLLT4-RB1 transcript found in case 6. The fusion is between the myeloid/lymphoid or mixed-lineage leukemia gene (MLLT4 on 6q27) and the retinoblastoma 1 gene (RB1 in 13q14). The former gene is usually found rearranged through a t(6;11)(q27;q23) leading to an MLL-AF6 in childhood AML; its finding is associated with poor disease outcome. The latter gene encodes a negative regulator of the cell cycle and was the first tumor suppressor gene discovered. This is the first time the two genes have been found together in a fusion transcript. Their role as well as the mechanism through which they act, being it the formation of a chimeric protein or loss of function for RB1, needs to be clarified.

The eight remaining fusions detected by the sequencing analysis and confirmed by PCR have not been reported before and their role in ESS needs further elucidation. Because these tumors were all classified as ESS by histology but showed no known ESS-specific fusion, they may represent new subgroups/entities under the big umbrella of ESS highlighting the presence of genetic heterogeneity in these tumors. Such heterogeneity is present in both HGEES and LGESS. In the
former group, it was demonstrated by the fact that not all HG showed YWHAE-fusion neither in the present series nor in the previous report by Scaillis et al. (2014). Both studies found that whereas YWHAE-rearranged ESS consistently shows HG cytomorphology, the reverse is not always true, i.e., not all uniform HGESS carry YWHAE-rearrangements. Furthermore, there remains a small group of LGESS which lack genetic rearrangements of known ESS-association fusion genes, indicating that additional, still unknown fusion transcripts may be characteristic of this tumor type and will be identified in the future. The expression profile, showing around 70 differently expressed genes, hints to the possibility that these tumors may belong to a new genetic subgroup. Further studies are necessary to obtain a more complete picture of ESS tumorigenesis. Because each approach has its pros and cons, it is mandatory to use a combination of techniques to avoid skewsness brought about by both false positive and false negative results.

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