The histone methyltransferase EZH2, an oncogene common to benign and malignant parathyroid tumors

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Abstract

Primary hyperparathyroidism (pHPT) resulting from parathyroid tumors is a common endocrine disorder with incompletely understood etiology. In renal failure, secondary hyperparathyroidism (sHPT) occurs with multiple tumor development as a result of calcium and vitamin D regulatory disturbance. The aim of this study was to investigate a potential role of the histone 3 lysine 27 methyltransferase EZH2 in parathyroid tumorigenesis. Parathyroid tumors from patients with pHPT included adenomas and carcinomas. Hyperplastic parathyroid glands from patients with HPT secondary to uremia and normal parathyroid tissue specimens were included in this study. Quantitative RT-PCR, western blotting, bisulfite pyrosequencing, colony formation assay, and RNA interference were used. *EZH2* was overexpressed in a subset of the benign and in all malignant parathyroid tumors as determined by quantitative RT-PCR and western blotting analyses. Overexpression was explained by *EZH2* gene amplification in a large fraction of tumors. *EZH2* depletion by RNA interference inhibited sHPT-1 parathyroid cell line proliferation as determined by tritium-thymidine incorporation and colony formation assays. *EZH2* depletion also interfered with the Wnt/ β -catenin signaling pathway by increased expression of growth-suppressive *AXIN2*, a negative regulator of β -catenin stability. Indeed, *EZH2* contributed to the total level of aberrantly accumulated transcriptionally active (nonphosphorylated) β -catenin in the parathyroid tumor cells. To our knowledge *EZH2* gene amplification presents the first genetic aberration common to parathyroid adenomas, secondary hyperplastic parathyroid glands, and parathyroid carcinomas. This supports the possibility of a common pathway in parathyroid tumor development.

Key Words

- ▶ Axin2
- ▶ β -catenin
- ▶ EZH2
- ▶ gene amplification
- ▶ hyperparathyroidism
- ▶ PRC2

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Introduction

Parathyroid disease with hypersecretion of parathyroid hormone and generally also hypercalcemia occurs in primary hyperparathyroidism (pHPT), due to growth regulatory disturbance in the parathyroid glands. pHPT is caused by a single, benign adenoma in ~85% of cases and parathyroid hyperplasia or multiple adenomas in

~15%, and very rarely by parathyroid carcinoma (<1%). In renal failure, secondary hyperparathyroidism (sHPT) occurs with multiple tumor development as a result of calcium and vitamin D regulatory disturbance (Marx 2000, Åkerström & Hellman 2004, Westin *et al.* 2009, Sharretts & Simonds 2010). Overexpression of the gene cyclin

D1 (*CCND1*) has been demonstrated in pHPT tumors (20–40%), due to gene translocation and oncogene activation (8%) and to other unknown mechanisms. Also secondary hyperplastic parathyroid glands (31%) and parathyroid carcinomas (90%) have been shown to overexpress cyclin D1 (Westin *et al.* 2009, Sharretts & Simonds 2010). *CCND1* constitutes a target gene for the Wnt/ β -catenin signaling pathway and in the absence of gene translocation, overexpression may in part be explained by pathway activation through aberrant accumulation of transcriptionally active (nonphosphorylated) β -catenin in the cytoplasm/nucleus (Shtutman *et al.* 1999, Lin *et al.* 2000, Björklund *et al.* 2007a, Westin *et al.* 2009). By immunohistochemical analysis, accumulation of total β -catenin has been observed in one study, as a frequent event in parathyroid adenomas and secondary hyperplastic parathyroid glands (Björklund *et al.* 2007b). Such accumulation has not been observed applying several other protocols, as recently summarized in Costa-Guda & Arnold (2013). Using western blotting analysis and a specific antibody (van Noort *et al.* 2002), the transcriptionally active (nonphosphorylated) form of β -catenin was found to be accumulated in parathyroid adenomas and secondary hyperplastic parathyroid glands, strongly implying activation of the Wnt/ β -catenin signaling pathway (Björklund *et al.* 2007b, Clevers & Nusse 2012). The accumulation of active β -catenin involved an aberrantly spliced internally truncated Wnt coreceptor LRP5. siRNA to *LRP5* was shown to reduce the nonphosphorylated active β -catenin level, transcriptional activation by β -catenin, and inhibited parathyroid tumor cell growth *in vitro* and in a xenograft SCID mouse model (Björklund *et al.* 2007c). The Wnt/ β -catenin signaling pathway has been shown recently to be activated also in parathyroid carcinomas, by aberrant accumulation of nonphosphorylated active β -catenin. This was caused by inactivation of the *APC* tumor suppressor gene, likely by promoter DNA methylation (Svedlund *et al.* 2010). Furthermore, inactivating somatic hereditary hyperparathyroidism type 2 (*HRPT2* (*CDC73*)) gene mutations are common in parathyroid carcinoma and may contribute to cyclin D1 overexpression (Woodard *et al.* 2005, Westin *et al.* 2009, Sharretts & Simonds 2010). Somatic inactivation of the multiple endocrine neoplasia type 1 (*MEN1*) tumor suppressor gene is frequently observed (~35%) in parathyroid adenomas (Lemos & Thakker 2008, Cromer *et al.* 2012, Newey *et al.* 2012).

Recently, it was demonstrated that the *HIC1* tumor suppressor gene was generally underexpressed in parathyroid adenomas and secondary hyperplastic parathyroid

glands and in parathyroid carcinomas (Svedlund *et al.* 2012). It was suggested that this involved repressive histone modification H3K27me_{2/3} rather than DNA methylation. Enhancer of zeste homolog 2 (*EZH2*) is the enzymatic part of the polycomb repressive complex 2 (PRC2), which is involved in many processes such as cell differentiation, proliferation, cell identity, and stem cell plasticity. PRC2 consists of several components, among them EED and SUZ12 promote PRC2 assembly and contribute to the H3K27 methyltransferase activity of *EZH2* (O'Meara & Simon 2012). *EZH2* is highly expressed in many solid tumors, it is considered to be an oncogene and amplification of *EZH2* has been observed in several cancers (Bracken *et al.* 2003, Saramaki *et al.* 2006). In lymphoma, heterozygous missense mutations at amino acid Y641 caused higher catalytic efficiency. In contrast, inactivating *EZH2* mutations have been found in myeloid neoplasms, supporting a tumor suppressor function (Chase & Cross 2011, Margueron & Reinberg 2011, Chang & Hung 2012). Recently, a link was established between *EZH2* and Wnt/ β -catenin signaling in the development of hepatocellular carcinoma (Cheng *et al.* 2011). *EZH2* was shown to mediate epigenetic repression of several Wnt antagonists, including the growth-suppressive *Axin2* which is known to negatively regulate the level of cytoplasmic β -catenin in the absence of Wnt ligand (Cheng *et al.* 2011, Clevers & Nusse 2012). *EZH2* may also interact directly with β -catenin as has been demonstrated in breast cancer (Shi *et al.* 2007, Li *et al.* 2009). In this study, we investigated a possible role of *EZH2* in parathyroid tumorigenesis.

Materials and methods

Tissue specimens

Parathyroid carcinomas ($n=5$), adenomas ($n=28$), and hyperplastic parathyroid glands secondary to uremia ($n=16$) from patients with pHPT and sHPT were acquired from patients diagnosed and operated upon in the clinical routine at Uppsala University Hospital and Martin Luther University of Halle-Wittenberg. Normal parathyroid tissue was obtained from glands inadvertently removed in conjunction with thyroid surgery where autotransplantation was not required ($n=1$) or as normal parathyroid gland biopsies in patients subjected to parathyroidectomy ($n=4$). The diagnosis of parathyroid carcinoma was unequivocal due to occurrence of metastases at diagnosis or follow-up. All tissues were intraoperatively snap frozen and cryosections were used in the analyses. Informed consent and approval of ethical committee was achieved.

Bisulfite treatment and pyrosequencing

Total DNA was extracted using AllPrep DNA/RNA Mini Kit (Qiagen) and bisulfite treatment was performed on 400 ng DNA using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Before pyrosequencing, a PCR was carried out with *EZH2* promoter primers: F, 5'-GTATT-TTGAGTGGGGTTGGTT-3'; R, 5'-CCCCCAACCAATCAA-3' (biotin labeled); sequencing primer, 5'-GTAGGAG-GGTTTGTGATTGGA-3'; sequence analyzed, YGGGYGTT-YGTTTYGYGTTTYGTTAATYGGGGYGGYGTTT.

The PCR included HotStarTaq Plus Master Mix (Qiagen), 10 pmol of each primer and bisulfite-treated DNA as template. PCR was carried out with an initial denaturation at 95 °C for 15 min, followed by 45 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and completed at 72 °C for 10 min. Pyrosequencing was done using 20 µl PCR product with the PyroMark Q24 system (Qiagen) according to manufacturer's instructions. The assay was validated to distinguish equal amplification of unmethylated and methylated DNA. This was done by analyzing a dilution series of bisulfite-treated CpGenome Universal, Methylated DNA (Millipore, Billerica, MA, USA; 100% methylated) and placenta DNA (~0% methylated).

RNA extraction and quantitative RT-PCR

DNA-free total RNA was extracted from pHPT tumors ($n=28$), sHPT tumors ($n=16$), parathyroid carcinoma ($n=5$), and normal parathyroid tissue ($n=5$) using AllPrep DNA/RNA Mini Kit and RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. Successful DNase-treatments were established by PCR analysis of all RNA preparations. RT of total DNA-free RNA was carried out with random hexamer primers using the RevertAid First, strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Quantitative RT-PCR was carried out on StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) using Maxima Probe/ROX qPCR Master Mix (Thermo Scientific) and Applied Biosystems TaqMan assays for *EZH2* (Hs01016789_m1), *SUZ12* (Hs00248742_m1), *EED* (Hs00537777_m1), *LRP5* (Hs_00182031_m1), *AXIN2* (Hs_00610344_m1), *CYCLIN D1* (Hs00765553_m1), and *GAPDH* (Hs02758991_g1). Each cDNA sample was analyzed in triplicate.

Copy-number variation

Total DNA was extracted using AllPrep DNA/RNA Mini Kit (Qiagen). Copy-number variation was determined

using Applied Biosystems TaqMan copy-number assays for *EZH2* (Hs02508122_cn and Hs02941342_cn) and *RNase P* reference (4401631) together with Maxima Probe/ROX qPCR Master Mix (Thermo Scientific) according to the manufacturer's instructions. The samples were run on StepOnePlus Real-Time PCR Systems and analyzed using CopyCaller v2.0 (Applied Biosystems). Each DNA sample was analyzed in quadruplicates with multiple runs and *EZH2* amplification was considered when both assays demonstrated this. DNA from placenta and normal parathyroid tissue were used as reference. Both *EZH2* PCR assays correctly detected four gene copies of chromosomal DNA from a control cell line with four *EZH2* gene copies (NA12519; Coriell Institute for Medical Research, Camden, NJ, USA).

Cell culture and transfection

sHPT-1 parathyroid tumor cells (2×10^5) were distributed onto 35-mm dishes in DMEM/10% fetal bovine serum (Sigma) and transfected in triplicates using Interferin siRNA transfection reagent (Polyplus Transfection; SAS, Illkirch, France). *EZH2* siRNA (HSS103462, Invitrogen), *LRP5* siRNA (sc-43900, Santa Cruz), and control nonsilencing siRNA (Qiagen) were transfected using 20 pmol. The cells were harvested after 120 h and RNA/protein was extracted. The cells were transfected for 48 h and then transfected again and further incubated for 72 h (Svedlund et al. 2012).

Cell viability, ³H-thymidine incorporation, and apoptosis

sHPT-1 parathyroid tumor cells were transfected with siRNA to *EZH2* (see above). The cell viability was measured after 2 h of incubation with the cell-proliferation reagent WST-1 (Roche Molecular Biochemicals), according to the manufacturer's instructions. For ³H-thymidine incorporation, 2 µl ³H-thymidine/well were added 12 h before harvest (NET027250UC from PerkinElmer, Norwalk, CT, USA). The medium was then aspirated and unspecifically incorporated ³H-thymidine was precipitated by adding 500 µl 10% trichloroacetic acid for 20 min with the plates on ice. The precipitated material was discarded and the procedure was repeated twice before lysing the cells with 500 µl 0.5 M NaOH and 0.5% Triton X-100/well. Radioactivity was monitored in a β-counter. Apoptosis was detected by using the Cell Death Detection ELISA Kit (Roche Molecular Biochemicals), according to the manufacturer's instructions. Alternatively, apoptosis was analyzed by poly(ADP-ribose) polymerase (PARP) expression and cleavage was detected by western blot

analysis. As a positive apoptosis control cells were incubated with 0.1 $\mu\text{g/ml}$ camptothecin.

Colony formation assay

sHPT-1 parathyroid tumor cells (2×10^5) were distributed onto 35-mm dishes in DMEM/10% fetal bovine serum (Sigma) and transfected in triplicates using FuGENE 6

transfection reagent (Roche Diagnostics) with 1 μg of control vectors or *EZH2* shRNA vectors (Fussbroich et al. 2011). After 24 h, cells were seeded at 4×10^3 in six-well plates and the following day 60 $\mu\text{g/ml}$ Hygromycin B were added to the culture medium. After 8 days, the selected cells were fixed with 10% acetic acid/10% methanol and stained with 0.4% crystal violet and the visible colonies were photographed. To monitor the effects of the shRNAs, the vectors were transiently transfected and protein extracts were prepared after 72 h followed by western blotting analysis.

Western blotting

Protein was extracted using Cytobuster protein extraction reagent (Merck Millipore) supplemented with Complete mini protease inhibitor cocktail tablets (Roche Diagnostics). Primary antibodies used were anti-EZH2 mouse MAB (17-662, Millipore) or EZH2 rabbit monoclonal D2C9 (5246, Cell Signaling Technology, Danvers, MA, USA), anti-active- β -catenin (van Noort et al. 2002; Upstate, Lake Placid, NY, USA, #05-665), anti-PARP rabbit polyclonal antibody (AB16661, Millipore), and anti- β -tubulin rabbit polyclonal antibody (sc-9104, Santa Cruz). After incubation with the appropriate secondary antibody, bands were visualized using the enhanced chemiluminescence system (GE Healthcare).

Immunohistochemistry

Paraffin-embedded specimens were stained as described (Björklund et al. 2007b) using an anti-EZH2 rabbit MAB (Cell Signaling Technology, Inc., EZH2 (D2C9), catalog no. 5246).

Statistical analysis

All data are presented as arithmetical mean $\pm$ S.E.M. Paired and unpaired *t*-test and Spearman's rank correlation were

Figure 1

EZH2 mRNA and protein expression in hyperparathyroid tumors. (A) Real-time quantitative RT-PCR analysis of *EZH2* in normal parathyroid tissues and parathyroid tumors. pHPT denotes parathyroid adenomas and sHPT secondary hyperplastic parathyroid glands. The relative expression level of the one normal parathyroid tissue that was obtained from glands inadvertently removed in conjunction with thyroid surgery was arbitrarily set to 1.0. The four normal parathyroid gland biopsies from HPT patients displayed relative *EZH2* expression levels of 0.8, 2.1, 2.2, and 3.1. (B) Western blotting analysis of EZH2. Tumors with relatively high mRNA levels (upper panel) or with mRNA levels in the normal range (lower panel). Samples no. 5, 7, 8, 14, and 15 displayed *EZH2* gene amplification. (C) Immunohistochemical analysis of EZH2. Paraffin-embedded sections were stained using a rabbit MAB (two upper panels) or without primary antibody (lower panel right). Western blotting of the same specimens is also shown.

used for statistical analysis. Statistical calculations were made in IBM SPSS Statistics, version 21. $P < 0.05$ was considered significant.

Results

EZH2 is overexpressed and the gene amplified in a subset of parathyroid tumors regardless of hyperparathyroid disease state

In order to analyze whether *EZH2* was differentially expressed in parathyroid tumors compared with normal parathyroid tissue specimens, quantitative RT-PCR was carried out. As shown in Fig. 1A, *EZH2* was generally significantly overexpressed in sHPT (3.9 ± 0.4) tumors and most apparent in parathyroid carcinoma (19.4 ± 4.5), when compared with normal parathyroid tissues (1.8 ± 0.4). For parathyroid adenomas, the mean value did not reach significance (pHPT: 3.5 ± 0.5), but 11 tumors showed higher mRNA levels than the highest expressing normal tissue. *EZH2* was significantly overexpressed in the parathyroid carcinomas compared with the adenomas and secondary hyperplastic parathyroid glands. No correlations between *EZH2* mRNA expression level and gland weight, serum PTH, serum calcium, or serum creatinine were found. Furthermore, western blotting analysis showed prominently increased protein expression of *EZH2* in parathyroid tumors with relatively high mRNA levels (Fig. 1B, upper panel). *EZH2* protein expression could also be detected in some pHPT and sHPT tumors with the relative *EZH2* mRNA levels in the normal range (Fig. 1B, lower panel). Immunohistochemistry analysis of *EZH2* revealed prominent cytoplasmic and nuclear immunoreactivity. An evenly stained level for most cells in all areas was observed in the adenoma that appeared more heterogenous in the carcinoma (Fig. 1C). mRNA levels were also determined for the PCR2 components *EED* and *SUZ12*. Only *SUZ12* showed significantly different expression, with a generally reduced level in secondary hyperplastic parathyroid glands (Fig. 2). The three parathyroid carcinomas with highest *EZH2* expression also showed overexpression of *EED*, and the two parathyroid adenomas with the highest *EZH2* showed highest expression of *SUZ12*. The very low expression of *EZH2* in the normal parathyroid tissue specimens was not associated with DNA methylation of the *EZH2* CpG island, as determined by quantitative bisulfite pyrosequencing analysis (data not shown).

Real-time quantitative PCR revealed relative amplification of the *EZH2* gene (four gene copies) in 29% of the pHPT tumors, 50% of the sHPT tumors, and 60% of the

Figure 2

Real-time quantitative RT-PCR analysis of PCR2 components *EED* and *SUZ12*.

parathyroid carcinomas (Table 1). Thirteen out of the 19 tumors with gene amplification displayed overexpression of *EZH2* mRNA and the three tumors analyzed by western blotting showed prominently increased protein expression (Fig. 1B, upper panel). Two tumors included in the western blotting analysis with gene amplification, and *EZH2* mRNA expression within the normal range showed weakly detectable or undetectable *EZH2* protein level respectively compared with normal tissue (Fig. 1B, lower panel). Some of the benign tumors and two of the carcinomas showed higher *EZH2* mRNA expression level without gene amplification. No correlations between *EZH2* gene copy number and gland weight, serum PTH, serum calcium, or serum creatinine were found.

Table 1 Copy-number variation of *EZH2* in parathyroid tumors

Tumor type	Number of tumors with <i>EZH2</i> amplification ^a	Total number of tumors analyzed	Percentage of tumors with <i>EZH2</i> amplification (%)
pHPT	8	28	29
sHPT	8	16	50
Carcinomas	3	5	60

^aAll displayed four gene copies.

Maintained expression of *EZH2* is required for parathyroid tumor cell growth

Next, we investigated whether *EZH2* could control growth of parathyroid tumor cells. siRNA to *EZH2* significantly inhibited proliferation and reduced cell viability of the human parathyroid tumor cell line sHPT-1, as determined by ³H-thymidine incorporation and WST-1 incubation

(Fig. 3A). The sHPT-1 cell line expresses parathyroid hormone and was established from a hyperplastic parathyroid gland removed at operation of a patient with sHPT (Björklund et al. 2007a). Colony formation assay was then performed to further evaluate a growth regulatory role of *EZH2*. sHPT-1 cells were stably transfected with *EZH2* shRNA expression vectors or control vectors (Fussbroich et al. 2011). The results showed a prominent reduction in the colony formation capacity in *EZH2* shRNA-expressing cells compared with control shRNAs, only a few very small colonies were seen in *EZH2* shRNA-expressing cells (Fig. 3C). Thus, *EZH2* depletion blocked the growth of parathyroid tumor cells. No apparent effect on apoptosis was detected after depletion of *EZH2* with siRNA or shRNA expression vectors, as determined by quantifying cytoplasmic histone-associated-DNA-fragments or by examining PARP protein expression and cleavage (Fig. 3B and D).

Figure 3

Cellular proliferation, viability, colony formation, and apoptosis on *EZH2* knockdown. Transient transfection of siControl and siEZH2 to the sHPT-1 parathyroid tumor cell line. Efficient knockdown of *EZH2* was obtained at the mRNA (4C) and protein level (Svedlund et al. 2012). (A) Effects on cell proliferation and cell viability were determined by ³H-thymidine incorporation (left panel) and WST-1 incubation (right panel). (B) Effects on apoptosis were analyzed by quantifying cytoplasmic

histone-associated-DNA-fragments (left panel) and induction of PARP expression and cleavage (right panel). (C) Colony formation assay. sHPT-1 cells were stably transfected with plasmids expressing two different shRNAs to *EZH2* (pCEP-shEZH2-1, pCEP-shEZH2-2) or two control shRNAs (pCEP-shluc, pCEP-shcontrol-1). (D) Western blotting after transient transfection of the shRNA vectors showing *EZH2* expression (upper panel) and induction of PARP expression and cleavage (lower panel).

EZH2 contributes to Wnt/ β -catenin signaling in parathyroid tumor cells

It has been reported that EZH2 can regulate Wnt/ β -catenin signaling indirectly by epigenetic repression of several Wnt antagonists, including the growth-suppressive Axin2, leading to enhanced level of transcriptionally active (nonphosphorylated) β -catenin (Cheng *et al.* 2011). In sHPT-1 parathyroid tumor cells, *AXIN2* expression was indeed increased under conditions with decreased *EZH2* level, and expression of *CYCLIN D1*, known to be regulated by β -catenin in parathyroid cells (Björklund *et al.* 2007a), was significantly reduced (Fig. 4A). Furthermore using an antibody specific for transcriptionally active (nonphosphorylated) β -catenin (van Noort *et al.* 2002), a reduced level of active β -catenin (15%) was observed in siEZH2-transfected sHPT-1 cells (Fig. 4B and C) compared with siControl (100%). As expected (Björklund *et al.* 2007c), siRNA to *LRP5* also reduced the active β -catenin level (30%) and furthermore β -catenin was undetectable after siEZH2 + siLRP5 cotransfection. Thus, EZH2 contributed to the total level of active β -catenin together with LRP5 in the parathyroid tumor cells.

Discussion

EZH2 gene amplification was observed in 19 out of 49 analyzed parathyroid tumors and was related to *EZH2* mRNA overexpression in 13 of these 19 tumors (Fig. 5, for summary and possible mechanisms). Importantly, western blotting analysis showed that it was possible to detect EZH2 expression also in tumors with or without gene amplification and with an *EZH2* mRNA expression level within the one for normal tissues. This may imply tight regulation of *EZH2* mRNA levels and perhaps relatively long half-life for EZH2 proteins in a subset of tumors. Some of the benign tumors and two of the carcinomas showed higher *EZH2* mRNA expression level without gene amplification, suggesting the involvement of other more indirect mechanisms. The c-Myc oncoprotein has recently been shown to positively regulate *EZH2* expression in early prostatic neoplasia, both directly as a transcriptional activator and indirectly by repression of miR-26a and miR-26b, that regulate *EZH2* mRNA at the post-transcriptional level (Koh *et al.* 2011). This mechanism may apply to the parathyroid as c-Myc overexpression has been reported for a subset of parathyroid adenomas and hyperplastic parathyroid glands from patients with HPT secondary to uremia (Björklund *et al.* 2007b). To our knowledge, *EZH2* gene amplification presents the first genetic aberration

Figure 4

Wnt/ β -catenin signaling on *EZH2* knockdown. (A) Transient transfection of siControl and siEZH2 to the sHPT-1 parathyroid tumor cell line and determination of *AXIN2* (left panel) and *CYCLIN D1* (right panel) expression by real-time quantitative RT-PCR analysis. (B) Western blotting analysis of transiently siRNA-transfected sHPT-1 cells using the anti-active β -catenin antibody. This antibody is specific for transcriptionally active (non-phosphorylated) β -catenin (van Noort *et al.* 2002). (C) Transient transfection of siControl, siLRP5, and siEZH2 and determination of *LRP5* and *EZH2* relative mRNA expression by real-time quantitative RT-PCR analysis.

Figure 5

Summary of the results and possible mechanisms. EZH2 protein overexpression caused by *EZH2* gene amplification and increased mRNA level or by indirect mechanisms such as EZH2 protein stability and by direct or indirect regulation of *EZH2* gene expression by the c-Myc oncoprotein (Björklund et al. 2007b, Koh et al. 2011). Deregulated increased EZH2 protein level could result in activation of the Wnt/β-catenin signaling pathway by repression of *AXIN2* (Cheng et al. 2011) and accumulation of transcriptionally active (nonphosphorylated) β-catenin, with increased expression of the β-catenin target gene *CYCLIN D1* (Björklund et al. 2007a) and proliferation. Repression of the *HIC1* tumor suppressor gene in parathyroid tumors involved EZH2 and H3K27 methylation (Svedlund et al. 2012).

common to a subset of parathyroid adenomas, secondary hyperplastic parathyroid glands, and parathyroid carcinomas. This further supports the possibility of a common pathway in parathyroid tumor development (Svedlund et al. 2012). Interestingly, somatic *EZH2* Y641N mutations were recently observed in parathyroid adenomas (Cromer et al. 2012), albeit at a very low frequency (two out of 193). Mutations at Y641 may result in enhanced catalytic methyltransferase activity and it seems that heterozygous mutations work in conjunction with WT *EZH2* to increase H3K27me3. This may be functionally equivalent to overexpression of *EZH2* (Sneeringer et al. 2010, Yap et al. 2011). *EZH2* can also act as a coactivator, independent of its role as repressor in the PRC2 complex and this activity of *EZH2* is oncogenic as demonstrated in prostate cancer cells (Xu et al. 2012).

Downregulation of *EZH2* inhibited proliferation and growth of sHPT-1 parathyroid tumor cells, as previously

observed for *LRP5* depletion and reduced level of non-phosphorylated active β-catenin (Björklund et al. 2007c). This in line with recent results showing reduced proliferation of primary parathyroid tumor cells and sHPT-1 cells by the global histone methyltransferase inhibitor 3-deazaneplanocin A (Miranda et al. 2009, Svedlund et al. 2012), which affects several different repressive histone modifications but also depletes *EZH2* (Fiskus et al. 2009). Overexpression of *EZH2* may result in deregulated expression of a large number of target genes, some of which contributes to parathyroid tumorigenesis. The tumor suppressor gene *HIC1* likely presents such a target gene since the aberrant underexpression of *HIC1*, observed in both benign and malignant parathyroid tumors, involved repressive H3K27me3 modifications and *EZH2* (Svedlund et al. 2012).

EZH2 may play a crucial role in metastatic parathyroid disease, as the gene was found to be significantly more expressed in the carcinomas in comparison with the benign parathyroid tumors.

Furthermore, it will be of interest to investigate whether *MEN1* gene inactivation and *EZH2* overexpression is functionally redundant or if both events contribute to parathyroid tumor development. It has been demonstrated that *MENIN*, encoded by the *MEN1* gene, can repress transcription through H3K27 methylation, in suppression of lung adenocarcinoma (Gao et al. 2009).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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