Author's response to reviews

Title: Prognostic relevance of Wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) promoter methylation in human breast cancer

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Author's response to reviews: see over
Prognostic relevance of Wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) promoter methylation in human breast cancer

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We thank the Editors for evaluating our manuscript in an external review process for publication in BMC Cancer. We appreciate the Reviewers’ valuable comments which helped us to further improve our manuscript. Responses to Reviewers’ comments are stated in the following remarks in a point-by-point manner. Changes in the manuscript text have been marked by underlining the word, sentence, or paragraph.

Summary of the revisions and new data included:

In summary, we have addressed all concerns raised by the two Reviewers, either by revision of the text or inclusion of novel calculations. Substantial revisions have been made to the statistical evaluations, for which we have performed new validation models.

Changes in Tables and Figures:

Previous Table 1: Following the Editor’s request, this table (patient characteristics) has now been omitted since it contained data that have been published previously (Veeck et al., Breast Cancer Research 2008). The remaining tables have been renumbered accordingly.

Table 3: The univariate analysis (log-rank tests) now contains a binary category for histological type (IDC vs. other) and the respective analysis results (previously Table 3).

Table 4: This table (Cox hazard estimates) has been revised, and now contains the categorization of all variables, the values that have been assigned to each category, and it indicates the category representing the reference value for a given variable. The variable “histological type” has been created as a binary variable (IDC vs. other) and the univariate and multivariate models have been recalculated accordingly. The previous univariate Cox regression analysis has been omitted since it was redundant to the univariate Kaplan-Meier analysis (Table 3). Results from a new adjusted Cox regression model (limit selection procedure) have been added.
Responses to Reviewers' comments

Reviewer 1: Mario Fraga:

Comment 1: A key issue is that the authors also found a positive correlation between DKK3 methylation and age and thus, it could be possible that the real association is between age of the patient and prognostic. An interesting aspect to explore here is why DKK3 is more prompt to be methylated in oldest breast cancer patients.

Authors’ response 1: The reviewer is right in that overall survival may be naturally related with patient age. In our analysis, we have therefore chosen “cancer-specific overall survival” as the endpoint, instead of the alternative commonly used term “unspecific overall survival” (as indicated in the Methods section). The latter approach would include deaths of any cause as endpoint, hence is very likely to be associated with advanced patient age. We have chosen only those patient deaths as events that were directly related to tumor disease. In this endpoint type of analysis, patient survival is not tightly associated with age, as it is demonstrated in Tables 4 and 5 (now Table 3 and 4). In fact, we detected only a very weak trend (p=0.094) of association between age and survival, for which we have included the potential confounder “age” in the univariate and multivariate Cox regression models (Table 5). In the latter model, DKK3 methylation was proven to be independent from age in predicting patient OS and DFS.

It may be interesting to know that if we included all events of death (cancer-related and non cancer-related) to the survival analysis, we still detect a significant association of DKK3 promoter methylation with adverse clinical outcome (P=0.0001; unmethylated: mean survival 157 months (95% CI: 142-173); methylated: mean survival 98 months (95% CI: 81-114) (see Figure 1). As expected, patient age became a significant factor (P=0.014) of impaired overall survival in such type of analysis as well, whereas other prognostic factors remained stable. Moreover, DKK3 methylation remained an independent factor in a multivariate model of such “unspecific overall survival” analysis (HR: 4.73; 95% CI: 1.58-14.18; P=0.006). Although DKK3 promoter methylation acts as prognostic factor in this type of survival analysis as well, the authors decided to account higher priority on uncovering the relationship between DKK3 promoter methylation and breast cancer-related deaths.
Fig. 1 Univariate Kaplan-Meier analysis of patient overall survival in relation to DKK3 promoter methylation. Overall survival was defined as time from primary surgery until any cause of death, and censored for patients alive at last contact. Patients harboring a methylated DKK3 promoter (red curve) show strongly shortened overall survival (P=0.0001).

Why DKK3 methylation is slightly more prevalent in older breast cancer patients than in younger patients cannot be explained here (mean age of DKK3-non-methylation is 54 years ± 14.1; mean age for DKK3-methylation is 60 years ± 12.2). We provided a possible explanation for this matter in the Discussion section, stating that methylation of genes can unspecifically occur during aging processes, and this may randomly overlay the effects of cancer-specific or cancer-driven methylation. However, it is also generally possible that DKK3 exerts a particular function in breast epithelia of older women after menopause, when the cycling exposure of the mammary gland to female hormones is strongly reduced. DKK3 (REIC) was originally identified as a mortalization-related gene whose loss of expression was associated with immortalization of cancerous cells (Tsuji et al., 2000), hence loss of DKK3 expression supported the escape from cellular senescence. Interesting in this context, DKK3 expression was found to be most abundant in normal senescent prostate epithelium (Untergasser et al., 2002) and also in organs with preponderance of growth-arrested post-mitotic cells, such as in heart and brain (Tsuji et al., 2000). After review of our DKK3 expression data in normal breast, we do not find a significant elevation of DKK3 mRNA in older patients, yet this does not rule out that DKK3 might not be implicated in cellular senescence of “older” breast epithelia after menopause. However, this possibility is merely speculation which we would rather not want to include in the Discussions section of the manuscript.

Comment 2: Up to now, Dickkopf-3 has been demonstrated to play a Wnt inhibitor role just in lung cancer (Yue et al., 2008. Carcinogenesis 29: 84-92). It has also been shown that Dkk-3 does not affect Wnt/catenin signaling in prostate cancer cells (Kawano et al., 2006. Oncogene 25: 6528–6537). Whether DKK-3 is or not
a Wnt inhibitor in breast tumours is something that needs to be further investigated. Moreover, it has not been published yet that WIF1 exerts a Wnt pathway inhibitor role of in breast cancer. So, inactivation of both genes does not necessarily involve an aberrant activation of the Wnt pathway in breast cancer.

Authors’ response 2: The Reviewer is right that in particular the role of DKK3 in Wnt signaling is still under debate. In prostate cancer cells DKK3 lead to apoptosis via JNK activation without the implication of β-catenin (Abarzua et al., 2005), whereas in malignant glioma DKK3 induced JNK-dependent apoptosis involving a decrease of β-catenin (Mizobuchi et al., 2008). In addition to the Wnt/catenin-inhibitory role in lung cancer (as mentioned by the Reviewer), DKK3 was also shown to inhibit Wnt signaling in mammary cells too, since DKK3 gene silencing by shRNA lead to a significant increase of β-catenin/TCF-dependent gene activity (Wang et al., 2008). To the authors believe, there is basic evidence that loss of DKK3 is involved in active Wnt signaling often found in mammary tumors. Despite, we had previously speculated a possible role of DKK3 essentially in non-canonical Wnt signaling, and since new contrary data have recently been published, we have now eliminated this speculative idea in the manuscript and refer to the inhibitory role of DKK3 on Wnt/β-catenin signaling only.

A Wnt-inhibitory role of WIF1 has also been recently published. Liu et al. (2008) showed e.g. that overexpression of WIF1 suppresses nuclear β-catenin activities and attenuates serum-stimulated phosphorylations of Akt and GSK3b in breast cancer MDA-MB-231 cells. Therefore, we believe there is also basic evidence that (epigenetic) inactivation of WIF1 contributes to active Wnt signaling in breast cancer.

Comment 3: The authors show that DKK-3 and WIF1 promoter methylation in patients are strongly correlated and that, at the same time, DKK3 alone is a good marker of prognostic. Thus, it must be explained and discussed why WIF1 is not.

Authors’ response 3: The Reviewer is right that this aspect has not been precisely explained in the manuscript. We intended to show that methylation of both genes is statistically connected, but given the high methylation frequencies of both genes this association is rather not surprising. Within our analysis, tumors having both genes unmethylated were counted as equal group as tumors having both genes methylated or having only one gene methylated. From a reasonable point of view rather from a statistical one, tumors having both genes unmethylated should be extracted from this analysis of regarding whether single or double methylation may be interrelated. In this case, the occurrence of methylation in either one or both genes would be 45:55%, i.e. if in breast tumorigenesis methylation of these genes occur, roughly half of the tumors gain methylation in one of the two genes only. To add precision to this point, we have deleted statements in the Results and Discussion section which stressed that in 63.3% of patients we have found an identical
methylation status (meaning both genes unmethylated AND both genes methylated), for this is inappropriate to compare.

It is DKK3 methylation that distinguishes the poor prognosis tumors better from the favorable prognosis tumors than WIF1 methylation. DKK3 methylation extracts the fraction of patients having poor prognosis, and whether WIF1 is methylated or not in the same tumors does not add further significance to this finding. This relation is illustrated in Figure 2, which shows the different survival characteristics according to WIF1 or DKK3 methylation. In this analysis, the DKK3-unmethylated tumors show a favorable prognosis, independent of whether WIF1 is methylated or not in these tumors (green and blue graphs). In contrast, DKK3 methylation shows the significant association with poor prognosis even in the absence of WIF1 methylation (red graph), so we conclude that from the half of the tumors that differ in their methylation status it is only the DKK3-methylated fraction that is implicated in poor survival.

![Figure 2](Image)

**Fig. 2** Univariate Kaplan-Meier analysis of patient overall survival in relation to WIF1 or DKK3 promoter methylation. Patients with unmethylated DKK3 in the tumor have a favorable prognosis independent of the WIF1 methylation status (green/blue curve). DKK3-methylated tumors, however, are associated with poor prognosis even in the absence of WIF1 methylation (red curve).

**Comment 4:** MSP results should be validated in some of the samples using bisulfite sequencing or pyrosequencing.

**Authors’ response 4:** The WIF1 and DKK3 MSP primers that were used for this study have already been published by other authors, and validation by bisulfite sequencing has already been demonstrated in other
reports (DKK3 in Lodygin et al., 2005; WIF1 in Urakami et al., 2006). Therefore, we found it dispensable to reproduce these validation results.

**Comment 5:** It would be interesting to provide expression data in order to assess the real biological role of DKK-3 and WIF1 hypermethylation in breast cancer.

**Authors’ response 5:** Loss of WIF1 or DKK3 protein and mRNA expression has been reported earlier. Wissmann et al (2003) analyzed WIF1 expression in human tumors, and reported loss of WIF1 protein, among other tumor entities, in 60% of breast carcinomas. Later, Ai and colleagues (2006) demonstrated that aberrant promoter methylation leads to loss of WIF1 expression, and they found a frequency of 67% of WIF1 methylation in breast carcinomas, which could be assigned to loss of WIF1 expression. Since we could confirm this methylation frequency in a much larger collective of breast cancers (63% loss in n=150 analyzed cases) it is reasonable to argue that loss of WIF1 expression is essentially if not completely mediated by promoter methylation in breast cancer. Neither loss of WIF1 expression (Wissmann et al., 2003) nor WIF1 methylation (our report) was associated with tumor stage, grade or lymph node status in breast cancer, thus being unlikely involved in tumor progression. In particular, WIF1 methylation occurred in 61.0% of pT1 tumors and showed no significant increase among tumor sizes (pT2 - pT4: 63.6%), which strongly suggests that WIF1 promoter methylation is an early carcinogenic event, at least in breast cancer. This conclusion has now been added to the Discussion section of the manuscript.

We have recently reported frequent loss DKK3 expression in breast cancer (Veeck et al., 2008). Comparably to WIF1, we found a quite concordant frequency of DKK3 methylation (61.3%) and loss of DKK3 expression (68.0%), and methylation was associated with loss of expression both on the RNA as well as the protein level. Again, neither loss of DKK3 expression nor DKK3 methylation was associated with clinical features of tumor progression such as tumor size, grade or lymph node invasion. DKK3 methylation was present in early invasive tumors as similar frequent as in advanced stage tumors, arguing for an early occurrence in breast tumorigenesis. This conclusion has now been added to the Discussion section of the manuscript.

**Literature:**
Reviewer 2; Mary Jo Fackler:

Comment 1: The statistical conclusions are in question due to a failure to correct for having performed multiple statistical comparisons. Despite the use of dozens of statistical tests, the authors claim in the Methods section that p values less than 0.05 “were considered significant” in the absence of appropriate correction. This is inappropriate.

Authors’ response 1: We thank Reviewer 2 for carefully reading the manuscript. In case of multiple statistical tests, the false discovery rate controlling procedure was applied (Benjamini and Hochberg, 1995; see Reference list at the end of the response letter). As regards Table 2 (m=8, q=0.05), p-values for the association between DKK3 methylation and age (P=0.007) and for the association between DKK3 methylation and WIF1 methylation (P=0.009) still remain significant on the 5% niveau. The manuscript has been completed accordingly and now reads as follows (Page #9; Materials and Methods section):

“Statistical analyses were completed using SPSS 14.0 (SPSS, Chicago, IL, USA). Differences were considered significant when P-values were below 0.05. To study statistical associations between clinicopathological factors and methylation status contingency tables and two-sided Fisher’s exact test were accomplished. In case of multiple statistical tests, the false discovery rate controlling procedure was applied. Survival curves were calculated...”.

Comment 2: The independence of DKK3 methylation as a prognostic factor is not established. See below.

Authors’ response 2: After extensive review of the data set we are still convinced that DKK3 methylation is an independent prognostic factor, as stated below.

Comment 3: To call DKK3 a “strong prognostic factor” and a “potent prognostic biomarker” in the Abstract seems misleading (even if we were to ignore the lack of statistical significance when appropriate corrections are made for multiple testing). In the univariate association study for predicting poor survival, the sensitivity and specificity were 46% (1-54%) and 97%, respectively. Thus, the “strong” test misses most of the patients who might benefit from an altered care pattern, and would not change the care of those receiving standard care. In the multivariate result, the confidence interval covers a broad range, which at its lowest is given by the authors to be a hazard ratio of 2.2. As discussed by Pepe et al (Am J Epi 159:882), hazard ratios in the range of 2 to 20 are not likely to separate the patients into clinically distinct treatment categories. That is, they are not clinically “potent”. A more convincing argument would need to be offered for the clinical advantages of the new tests.
Authors’ response 3: The Reviewer is right that the single assessment of this marker would unlikely separate patients into distinct treatment categories. However, this claim was not the intention of the authors’ report. We report on the association of DKK3 methylation with poor patient survival in breast cancer, which demonstrates that patients showing DKK3 methylation in the tumor have an unfavorable prognosis. As stated in more detail in response 11, we believe that DKK3 methylation is merely one candidate of a panel of genes that are associated or possibly even implicated in shorter survival in terms of developing a more aggressive tumor subtype. It is the aim of our currently ongoing studies to find such marker genes whose methylation further extracts the worst prognosis fraction from those patients harboring DKK3 methylation.

Comment 4: The statistical test applied was insufficient to conclude that DKK3 methylation was an independent variable associated with prognosis. The Abstract does not report that a prognostic model containing DKK3 methylation status can out-perform a prognostic model lacking this new variable. One method by which to do this compares the loglikelihood values of a model with and without the variable. Unless the two models differ significantly, one would assume that DKK3 was merely sharing the information already available from older, established clinicopathological parameters. The beta value provided in a given Cox model does provide a measure of the strength of the prognostic vector assigned by the model to the variable, but one needs to compare two models in order to determine whether the model has attributed some of the information that was already available from other variables. In order to do this, one needs to compare the new model to one specifically lacking only the variable of interest, in which, obviously, the model could not be attributing the already-available information to the new and herein-absent variable.

Authors’ response 4: We have now tested the robustness of the presented Cox regression model by further statistical evaluation. First, we have assessed and compared the -2 log likelihood functions of all covariates for overall survival and disease-free survival. In this analysis we obtained similar log-minus-log functions of all categories that were significant in the Cox model. To indicate this validation procedure, the following statement has been added to the Methods section: “The proportionality assumption for all variables was assessed with log-negative-log survival distribution functions”. Second, we have calculated adjusted Cox regression models in addition to the presented global Cox model. In these adjusted models using the reverse and also forward selection procedure we could confirm that DKK3 was an independent prognostic factor of overall survival in breast cancer, since the covariate was included in the final models after forward and backward selection. The covariate disease-free survival also remained in the model after forward selection, but was excluded after the backward selection procedure. Therefore we conclude that the prognostic potency of DKK3 methylation for disease-free survival is not as independent as it is for overall survival, possibly due to overlaying information with other prognostic factors such as a positive node status. The results from these adjusted Cox regression models after backward selection have now been added to the global Cox
regression model in Table 4. Since the adjusted models were calculated as a validation of the global Cox model, we stated the original numbers from the global model in the Abstract and Results section.

Comment 5: The statistical treatments of histological type in the Cox analysis appears incorrectly performed. According to Table 5, histological type was treated as an ordinal variable. Histological type is ungrouped, presumably leaving three data categories to be assigned numerical values by the software. Cox analysis classically fails with anything other than binary data types, and thus it is usually advisable to “dummy up” these complex variables to convert them into binary variables. Whatever category is assigned to the highest ordinal value is assumed to perform exponentially worse or exponentially better than the middle ordinal value, when each is compared to the lowest ordinal value. This is a silly assumption. For example, a clumsy treatment of “histological type” could yield a poor performance in the Cox model – which seems plausible due to the low HR of 1.05 for this variable.

Authors’ response 5: We thank the Reviewer for this advice of inappropriate handling of histological type in the Cox model. Following the advice, we have now created a nominal binary variable for histology consisting of IDC versus any other histological type, and recalculated the models accordingly. However, this did not significantly alter the results from these models, but improved precision.

Comment 6: The association of DKK3 methylation and overall survival is rendered largely irrelevant by the lack of statistical association between DKK3 methylation and disease-free survival (see Figure 5). Thus, it seems to be a random coincidence.

Authors’ response 6: In Figure 5, the univariate analyses of WIF1 and DKK3 with overall and disease-free survival are presented. In this type of analysis, DKK3 methylation actually is significantly associated with DFS, yet with weaker significance than OS (p=0.037). Clinically, DFS merely reflects the benefit of adjuvant therapeutic treatment, and its assessment is dependent on the definition of appropriate endpoints, such as recurrence/relapse, second primary cancer (locally/any site), or distant metastases [a matter addressed in: Chua YJ, et al: Annals of Oncology 2005, 16:1719-1721.]. However, this definition variability implies a spectrum of possible interpretations considering patient outcome. To avoid such potential principal bias we used a comprehensive definition of DFS endpoint, including all cancer-related events of disease, except death. Further stratified analyses of our cohort will show whether DKK3 methylation might be associated with a single aspect of disease, e.g. local or distant relapse. In our belief, the association of DKK3 methylation with poor OS is an obvious indicator of this implication. Unfortunately, at this time we are not able to perform a subanalysis with regard to chemotherapeutic treatment since these data are not yet completely available.
**Comment 7:** The statistical treatments of age in the Cox analysis is unclear, but is potentially important because of the discovered association of age and methylation status. According to Table 5, the continuous variable age was treated as “ordinal”. This is ambiguous. If age was “dummied up” into a binary variable (into two categories) by dividing the data at the median, this would not be an “ordinal” dataset. To treat age as an ordinal variable implies more than two categories, yet just how the Cox proportional hazards assumptions would handle ordinal data having three or more categories is not explained. The authors should clearly explain why age was not handled in binary form as were the other continuous variables (such as tumor size).

**Authors’ response 7:** The authors are sorry for this error in the table footnote. In fact, age had been treated as a continuous variable in the Cox models and histology had been treated as ordinal variable consisting of three categories, but unfortunately the footnote indicating this was wrong (as well as the categorization itself). In the revised version, we have now included age as a binary nominal variable dividing patient groups at the median age, and we also included histology as a binary “dummy variable” to the Cox models, according to the univariate log-rank analysis in Table 3 (previously Table 4).

**Comment 8:** The grouping of all three types of primary breast carcinoma (ductal, lobular, and other) is problematic. These are very different tumor type. It might not be reasonable to expect a prognostic behavior to be manifest similarly among the three categories. The Cox model and the univariate models DO expect similarity (“proportionality”) of behavior from a variable among the subclassifications of the patient set. Stratifying the data and performing a separate analysis for each distinct category is one way to handle such issues.

**Authors’ response 8:** Unfortunately, the number of cases in our cohort is not large enough to perform a reliable stratified subanalysis among the different histological types of breast cancer. Despite, we have analyzed the new binary variable of histological type in a correlation analysis versus all other clinicopathological factors, and this did not reveal a significant association of histology (IDC/other) with any of the other analyzed factors. Moreover, this histology variable neither showed significance in the Kaplan-Meier analysis nor in the Cox hazard models, for why we believe it being not confounding in our study.

**Comment 9:** The Methods section does not state that all diagnoses were reviewed by coding the diagnostic samples and submitting them to a pathologist expert in the disease.

**Authors’ response 9:** The Reviewer is right. This specification has now been added to the Methods section.
Comment 10: The statistical methods state that “only patients for whom the status of all variables was known were included in the proportional hazard models”. How many remained suitable is unclear.

Authors’ response 10: The Reviewer is right. Since the data sets of the included variables were not complete for all the patients, the number of patients remaining in the multivariate model was reduced to n=103, as compared to n=125 in the univariate model. This figure has now been added to the Methods section.

Comment 11: The specificity of the finding was not seriously examined. It is possible, for example, that the tumors having worse prognosis are methylated with some specificity at many genes, and that DKK3 is merely one of them. Without having examined the methylation of a large panel of gene (i.e., many more than two genes), one cannot state that the choice of gene, nor the function of the gene, has any special relation to the prognostic associations sought.

Authors’ response 11: The Reviewer’s suggestion is truly what we believe, namely that DKK3 is only one of a set of genes that are related with poor prognosis in case of hypermethylation. Neither the high frequency of 61% nor the lack of association with any clinicopathological factor would be reasonable to argue that DKK3 methylation alone leads to poor patient survival. In our laboratory, we have subsequently analyzed a large panel of genes, and hypermethylation of some particular genes show similar yet weaker associations with survival in breast cancer. If we combine those gene candidates showing survival associations in a marker panel, we are able to extract the patient fraction having the worst prognosis. In detail, DKK3 is the most potent of those candidate genes, but nevertheless when methylated shows better prognosis if other candidates are unmethylated in the same tumor (in contrast to WIF1 methylation reported in this study). Thus, among the DKK3-methylated tumors there are still tumors with a better and such with a worse prognosis. Tumors showing methylation in all “prognostic genes” (currently 4) show the worst prognosis. Since we want to report this gene panel in a future report including far more patient samples we here aimed at the single presentation of DKK3 as prognostically relevant gene in breast cancer.

Comment 12: The conclusion in the Abstract that WIF1 and DKK3 have different prognostic associations (“only DKK3 methylation proves …”), is not established with a statistical direct comparison of the two markers’ associations. The conclusion thus exceeds the data. The more likely explanation for the behavior of these two markers is that due to lack of adequate statistical power, this is an apparent, rather than an actual, difference.
Authors’ response 12: WIF1 methylation was not a significant factor in the univariate analysis, in contrast to DKK3 methylation. Therefore, we had not calculated the Cox proportional hazard models for WIF1 methylation. When we do so, WIF1 methylation is not a factor of prognostic significance, neither in models containing only WIF1 methylation nor in models containing both WIF1 and DKK3 methylation. In detail, in a global Cox model of OS containing both genes’ methylation, DKK3 methylation shows advanced prognostic significance (HR = 16.0; P=0.008), whereas in such model of DFS its significance becomes slightly reduced (HR = 2.3; P=0.072). To the authors’ believe the different behavior of these two markers thus seems not to be a random or apparent difference.

Comment 13: To “conclude that DKK3 may exert important tumor suppressive functions” exceeds the data. The data concern only an association with prognosis. Associations cannot be used to conclude causative relationships unless all other alternative explanations can be excluded.

Authors’ response 13: The reviewer is right that this aspect has been misleadingly presented. This conclusion was originally presented three times: Once in the Abstract, where it now has been omitted; a second time in the Discussion, where we concluded that DKK3 may exert tumor suppressive functions based on the experimental reports cited within this passage (Roman-Gomez et al., 2004; Kobayashi et al., 2002; Lodygin et al., 2005). Since the conclusion is not drawn from our outcome analysis but from other experimental evidence we decided to leave this statement here. The third mentioning of this conclusion was stated in the very last sentence of the “Conclusions” section. We here intended to support the evidence-based hypothesis that DKK3 may act tumor suppressive, rather than raise this hypothesis ourselves based on our outcome analysis (which we admit would have been simply false). To meet this request, we have deleted this statement from the Conclusions section.

Comment 14: The statistical treatments of the nodes and histological grade are unclear. Grades 1 and 2 were grouped, but it is unclear that this would be appropriate. Similarly, N1-3 are grouped, without comment.

Authors’ response 14: The grouping of Grade 1 and Grade 2 tumors was performed to compromise the underrepresentation of G1 tumors in our cohort (9%, see previous Table 1). We were interested in seeing whether methylation was associated with indicators of progression, i.e. with higher grade (G3) tumors versus lower grade (G1-G2) tumors. If we had found a significant association of methylation within this grouping of grade, we would have also presented the values of the separate analysis from grade being analyzed with three categories. For WIF1 and DKK3 methylation such correlation analysis yielded negligible p-values of p=0.586 in both cases. In fact, grade consisting of three categories performed very similar to the grouped variable in univariate Kaplan-Meier analysis, as well as in the Cox hazard models.
Regarding lymph node invasion, we were also interested in the progression-indicating subgroups “node negative” (pN0) versus “node positive” (pN1-3). Again, if we had found a significant association of methylation with lymph node status it would have been interesting to sub-analyze whether this association remains significant within the number of positive lymph nodes. Since this was not the case, we found it appropriate to present lymph node status in the analyzed form as “positive” versus “negative”. The grouping modalities of tumor size, lymph node status and grade have now been added to the Materials and Methods section (Statistical evaluations).

**Comment 15:** Footnote #2 of Table 5 seems to state that only the HR values of the multivariate analysis were obtained from the Cox hazard estimates. How were the HR values of the “univariate” analyses obtained?

**Authors’ response 15:** The Reviewer is right. This error has now been eliminated. HR values of both analyses have been obtained from the Cox hazard estimates; hence this footnote is self-explanatory and has been omitted.

**Comment 16:** The Abstract’s paragraph on Methods should state that the methylation was interpreted in a binary, or qualitative, fashion. An alternative would be to use a quantitative scale.

**Authors’ response 16:** This specification has now been added to the Abstract’s Methods section.

**Comment 17:** In the Abstract, providing the brand of software is presumably irrelevant, for all competent statistical packages should give the same answers. If the authors feel that the choice of software is a relevant detail deserving of mention in the Abstract, they should convey in the Abstract why they chose one package over another. If, instead, they feel that the choice was irrelevant to the results obtained, the brand need not be mentioned except in the formal Methods section. In the Abstract, mentioning the types of statistical tests is indeed informative, but is most informative for the reader when placed adjacent to the p values in the Results paragraph rather than in the Methods.

**Authors’ response 17:** We have eliminated the Software brand from the Abstract, for it is truly irrelevant. However, since the main message of the manuscript is built on statistical analyses, we decided to leave the mentioning of applied statistical tests in the Abstract’s method section.

**Comment 18:** The Abstract mentions a statistical test finding that “WIF1 methylation was significantly correlated with methylation of DKK3”, yet the statistical methods listed (Fisher test) is a test of association,
not a test of correlation. In the formal Methods section, the correct term, “associations”, is used by the authors.

**Authors’ response 18:** The Reviewer is right. The wrong use of “correlation” instead of “association” has been eliminated in the manuscript.

**Comment 19:** In the Abstract, the Kaplan-Meier equations are seemingly depicted as a statistical evaluation, which they are not. Instead, it was the log-rank test that the authors used to statistically evaluate the lifetable data in a univariate manner.

**Authors’ response 19:** This concern has been addressed by now correctly stating that this type of analysis has been statistically assessed by log-rank tests (see Abstract section).

**Comment 20:** The term “clinicopathological patient parameters” is redundant, for “clinicopathological parameters” is adequate.

**Authors’ response 20:** The Reviewer is right. We have changed the term “clinicopathological patient parameters” into “clinicopathological parameters” throughout the manuscript.

**Comment 21:** The word “Univariate” in the Results of the Abstract should be revised to read, “In univariate analysis“.

**Authors’ response 21:** This term has been corrected.

**Comment 22:** Proper hyphenation is needed and aids readability. “Patients with DKK3 methylated tumors”, for example, should read, “patients with DKK3-methylated tumors”, since the “patients specifically with DKK3” had not exclusively “methylated the [Rest missing in report].

**Authors’ response 22:** Thank you for this advice. We have revised the hyphenation throughout the manuscript and added hyphens where appropriate.

**Literature:**