High Expression of Wee1 Is Associated with Poor Disease-Free Survival in Malignant Melanoma: Potential for Targeted Therapy

Gry Irene Magnussen¹, Ruth Holm¹, Elisabeth Emilsen¹, Anne Katrine Ree Rosnes¹, Ana Slipicevic¹², Vivi Ann Florenes¹*  
¹ Department of Pathology, The Norwegian Radium Hospital, Oslo, Norway, ² The Wistar Institute, Philadelphia, Pennsylvania, United States of America

Abstract

Notoriously resistant malignant melanoma is one of the most increasing forms of cancer worldwide; there is thus a precarious need for new treatment options. The Wee1 kinase is a major regulator of the G2/M checkpoint, and halts the cell cycle by adding a negative phosphorylation on CDK1 (Tyr15). Additionally, Wee1 has a function in safeguarding the genome integrity during DNA synthesis. To assess the role of Wee1 in development and progression of malignant melanoma we examined its expression in a panel of paraffin-embedded patient derived tissue of benign nevi and primary- and metastatic melanomas, as well as in agarose-embedded cultured melanocytes. We found that Wee1 expression increased in the direction of malignancy, and showed a strong, positive correlation with known biomarkers involved in cell cycle regulation: Cyclin A (p < 0.0001), Ki67 (p < 0.0001), Cyclin D3 (p = 0.001), p21Cip1/WAF1 (p = 0.003), p53 (p = 0.025). Furthermore, high Wee1 expression was associated with thicker primary tumors (p = 0.001), ulceration (p = 0.005) and poor disease-free survival (p = 0.008). Transfections using siWee1 in metastatic melanoma cell lines; WM239WTp53, WM45.1MUTp53 and LOXWTp53, further support our hypothesis of a tumor promoting role of Wee1 in melanomas. Whereas no effect was observed in LOX cells, transfection with siWee1 led to accumulation of cells in G1/S and S phase of the cell cycle in WM239 and WM45.1 cells, respectively. Both latter cell lines displayed DNA damage and induction of apoptosis, in the absence of Wee1, indicating that the effect of silencing Wee1 may not be solely dependent of the p53 status of the cells. Together these results reveal the importance of Wee1 as a prognostic biomarker in melanomas, and indicate a potential role for targeted therapy, alone or in combination with other agents.

Introduction

Malignant melanoma is the second most increasing form of cancer in Norway, following prostate (men) and lung cancer (women) [1]. Whereas the prognosis is good when detected early, there are no curative treatments once the cancer has spread to distant organs (stage IV). Thus, there is a desperate need for new and more effective treatment options.

The cell cycle is the orderly series of events leading to cell division, and is regulated by the assembly and activation of complexes of CDKs and cyclins, which again triggers the transition between each of the four phases: Gap 1 (G1), DNA synthesis (S), Gap 2 (G2) and mitosis (M). During these events, DNA damages may arise both as a consequence of normal metabolic activity and due to environmental factors, and division may be arrested/delayed at three major DNA damage checkpoints (G1/S, intra- S and G2/M) before cell division. The G1/S checkpoint is largely controlled by p53, a tumor suppressor protein which function is impaired/lost in the majority of cancers, thus compromising this checkpoint. Hence most cancer cells exposed to DNA-damage rely on the S- and G2/M checkpoints for repair to occur. Encountering the S-phase checkpoint, genomic insults cause cells to slow down cell cycle progression rather than being arrested, rendering the G2/M checkpoint to ultimately halt the cell cycle progression [2]. Central in regulating the transition between the G2 and M phases is Wee1-like protein kinase (Wee1), a tyrosine kinase [3]. Wee1 negatively regulates entry into mitosis by phosphorylating the Tyr15 residue of Cyclin-dependent Kinase 1 (CDK1, also known as CDC2), thus inactivating the CDK1/cyclin B complex and arresting the cell cycle.

In addition to being a key regulator of the G2/M checkpoint, Wee1 also plays an active role in stabilizing the genome in the S-phase. By suppressing CDK2 activity during DNA synthesis, Wee1 prevents unscheduled initiation of replication that may potentially lead to DNA lesions [4].

Kinases, such as Wee1, represents potential therapeutic targets, however, their expression varies in different types of tumors. Over-expression of Wee1 has previously been reported in osteosarcoma, glioblastoma and breast cancer [5-7]. Under-expression, on the other hand, has been described in non-small-cell lung cancer [8]. Cell lines showing an enhanced level of Wee1 have also been demonstrated to be more sensitive to treatment with siWee1 [6].
Due to many promising in vitro results, the Wee1-inhibitor MK1775 have very recently been included in two phase I clinical trials both as mono-therapy and in combination with either 5-fluorouracil [9] or topotecan/cisplatin [10].

In the present study, we demonstrate for the first time that Wee1 is up-regulated in human malignant melanomas as compared to normal melanocytes and benign nevi, and that high expression of Wee1 is associated with poor disease-free survival and markers of increased tumor cell proliferation. Our in vitro results further revealed a reduced amount of viable cells, accumulation of cells in G1/S or G2-phase and double-strand DNA breaks following transfection with siWee1 in both p53 wild-type and mutated melanoma cell lines. Together our results indicate a role of Wee1 in proliferation and genomic stability in malignant melanoma, thus potentially making the kinase an eligible therapeutic target.

Materials and Methods

Specimens

Formalin-fixed, paraffin-embedded tissue sections from 108 primary malignant melanomas (75 superficial spreading (SSM) and 33 nodular melanomas (NM)), 23 metastases and 10 benign nevi (7 combined, 2 combined intradermal and 1 intradermal) were randomly collected from the archives of The Norwegian Radium Hospital and regional hospitals. Clinical follow-up was available for all patients. The Regional Committee for Medical Research Ethics South of Norway (S-06151) and The Social and Health Directorate (06/2733) approved the current study protocol.

Immunohistochemical analysis

Three-μm sections made from formalin-fixed paraffin-embedded tissues were used using the Dako EnVision™ Flex+ System (K8012, Dako Glostrup, Denmark). Deparaaffinization, rehydration and target retrieval were performed in one operation in a Dako PT-link and EnVision™ Flex target retrieval solution with high pH. To block endogenous peroxidase the sections were treated with Dako EnVision Peroxidase Block for 5 minutes. Thereafter, the sections were incubated in 0.48% dispase II (Invitrogen) at 4°C for 60 minutes. Next, the sections were incubated in 0.05% trypsin for 5 min in 37°C. The suspensions were diluted in 254CF medium (Invitrogen) and serially filtered through 40 μm cell strainers (Becton Dickinson, Franklin Lakes, NJ). The cells were plated in the T25 flask and cultured until confluence was reached. Differential trypsinization was used for first passage in order to obtain a pure melanocyte culture. Once confluent, 2×10⁶ melanocytes were harvested using EDTA, embedded in 200 μL 1.5% agarose and fixed in 10% neutral buffered formalin for 1 hour, and processed by routine histological methods.

Cell lines and Growth conditions

The human metastatic melanoma cell lines WM45.1 and WM239 were kindly provided by Dr. Meenhard Herlyn (the Wistar institute, Philadelphia, USA) [16,17]. The LOX cell line was established from a lymph node biopsy of a melanoma metastasis, at the Norwegian Radium Hospital (Oslo University Hospital, Norway) [18]. All cell lines were maintained in RPMI-1640 medium (LONZA, Verviers, Belgium) supplemented with 5% Fetal Calf Serum (Biochrom, KG, Berlin, Germany) and 2 mM L-glutamine (LONZA, Verviers, Belgium). The cells were grown in monolayer culture at 37°C in humidified conditions containing 5% CO₂ and 95% air.

Small interfering RNA (siRNA) transfection

All cell lines were plated out in either 6-well plates (1.5×10⁵ cells/well) or in 96-well plates (5×10³ cells/well) 24 hrs in advance of the transfection. The cells were transfected with 10 nM siRNA targeting Wee1 (OligioID: ‘VHS50841’) or RNAi negative control duplexes (Negative Control LOW GC, 12935-200) using Lipoctectamine™ RNA MAX transfection reagents (all reagents from Invitrogen corporation, CA, USA).

MTS assay

Five thousand cells per well were seeded in 96-well plates and left to attach overnight, before siRNA transfection for the indicated time.

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, WI, USA), in which the capacity of the cells to convert MTS salt into a brown formazan product was measured. Absorbance was measured at 490 nm using ASYS UVM340 96-well plate reader.

Statistical analysis

Statistical analysis was performed using of SPSS version 18.0 (Chicago, IL). The relationship between the expression level of Wee1 and tumor thickness was evaluated non-parametrically using the Mann-Whitney two sample test. Comparison between Wee1 expression and Ki-67, Cyclin A-, D1-, D3, p21<sup>CDKN1A</sup>, p27<sup>kip1</sup>, p53 as well as SSM and NM, was conducted by the use of chi-square tests. Kaplan-Meier survival estimate was used to evaluate the impact on survival.

Melanomas isolation

Normal melanocytes were isolated from human foreskins. Briefly, foreskins derived from circumcisions of newborns were washed with Hank’s balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA). Excess adipose tissues were removed, and the skin specimens were cut into approximately 0.5×0.5 cm² pieces and incubated in 0.48% dispase II (Invitrogen) at 4°C. After 18 hours, the epidermis was manually removed from the dermis, cut and digested in 0.05% trypsin for 5 min in 37°C. The suspensions were diluted in 254CF medium (Invitrogen) and serially filtered through 40 μm cell strainers (Becton Dickinson, Franklin Lakes, NJ). The cells were plated in the T25 flask and cultured until confluence was reached. Differential trypsinization was used for first passage in order to obtain a pure melanocyte culture. Once confluent, 2×10⁶ melanocytes were harvested using EDTA, embedded in 200 μL 1.5% agarose and fixed in 10% neutral buffered formalin for 1 hour, and processed by routine histological methods.

High Wee1 Correlates with Poor Outcome in Melanoma

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Trypan blue dye exclusion test

Cells treated with SiCtrl or SiWee1 were harvested using trypsin/EDTA (LONZA), along with medium containing floating cells. After centrifugation, the cell pellet was resuspended in PBS containing trypan blue (Merck, Stockholm, Sweden). Viable (dye excluding) and trypan blue stained dead cells were counted.

Cell Death Detection ELISA<sup>plus</sup>

Determination of cytoplasmic histone-associated-DNA-fragments was assessed using a commercially available kit (Roche Diagnostic GmbH, Mannheim, Germany), following the manufacturers instructions. The presence of histones in cytoplasm is indicative of apoptosis. The ELISA signal was quantified by measuring the absorbance at 405 nm (reference 495 nm), using ASYS UVM340 96-well plate reader (Fisher Scientific, Oslo, Norway).

Flow cytometric cell cycle analysis

Cells were harvested by trypsinization and washed 1 x in PBS. Cell pellets containing approximately 10<sup>6</sup> cells were re-suspended in 1 mL 70% ice-cold methanol and left to fixate for a maximum of 24 hrs. Fixed cells were washed 1 x in PBS, and stained with a solution containing 2 μg/mL Hoechst 33258 in PBS. Flow cytometric analysis was performed using LSR II UV laser (BD biosciences, San Jose, CA).

Western blot analysis

Cells were harvested using a rubber policeman, washed once in 1 x PBS, and then lysed in ice-cold NP-40 Lysis buffer (1% NP-40, 10% glycerol, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 100 mM NaF, 1 mM Aprotinin (0.02 mg/mL), Phosphatase inhibitor cocktail 1 (10 μL/mL), Phosphatase inhibitor cocktail 2 (10 μL/mL), PhenylMethaneSulfonyl Fluoride (PMSF) (1 mM), Leupeptin (0.02 mg/mL), Pepstatin (0.02 mg/mL) and Sodium vanadate (1 mM) (Sigma-Aldrich, St. Louis, MO)). Bradford (Bio-Rad Laboratories AB, Sundbyberg, Sweden) analysis was performed for protein quantification, and 25 μg protein/lane was resolved in SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a PDVF immobilon membrane (Millipore, Bedford, MA). To ensure even loading, filters were stained with naphtholblue black (Sigma-Aldrich) and later re-stained with α-tubulin. The membranes were blocked in 5% non-fat milk in TBST (150 mM NaCl, 25 mM Tris-CI, (pH 7.5), 0.01% Tween 20), and probed with primary antibodies at 4°C overnight, with gentle agitation. Primary antibodies Caspase 3 (#9662/#9664 (even mix)), Caspase 8 (#9746), Caspase 9 (#9502), Cyclin B1 (#4138S), Cyclin D3 (#2936), p21<sup>cip1/Akip1</sup> (#2946), p-p38 Thr180/Tyr182 (#4631) and PARP (#9532), were purchased from Cell Signaling (Beverly, MA). α-tubulin (DMIB) was acquired from Calbiochem (Nottingham, UK), whereas Cyclin A (sc-751), p53 (sc-126) and Weel (sc-5285) were obtained from Santa Cruz (Santa Cruz, CA). γ-H2AX (#05-636) and pCDK1<sup>Tyr15</sup> (ab47594) antibodies were acquired from Millipore and Abcam (Cambridge, England, respectively). Membranes were thereafter washed 3 x 10 minutes in TBST. Membranes were hybridized with an appropriate secondary antibody (HRP-conjugated anti-rabbit or anti-mouse IgG antibodies (Promega)) for 1 hr at room temperature, with gentle agitation, and then washed in TBST for 3 x 10 minutes. Protein bands were detected after first incubating the membranes with ECL-plus (GE Healthcare, Chalfont St Giles, UK) for 5 minutes, and then exposing them to X-ray films.

Results

Increased expression of Wee1 in melanoma

High protein expression of Wee1 has previously been reported in human cancers [5–7]. Since the status of Wee1 expression in melanomas has not been extensively studied, paraffin-embedded tissue from a panel of benign nevi and primary- and metastatic melanomas, in addition to a sample of cultured melanocytes, were analyzed for Wee1 protein expression by immunohistochemistry. As illustrated in Figure 1A, protein expression of Wee1 was hardly detectable in the nucleus of the cultured melanocytes, however brown granules were seen in the cytoplasm, most likely due to melanin. Furthermore, as demonstrated in Table 1 and illustrated in Figure 1A, a heterogeneous Wee1 staining pattern was observed in the vast majority of the tumor samples. However, the percentage of positive cells varied in tissues of different stages. Based on distribution, positive immunoreactivity in ≥10% of the tumor cells was used as cut-off to discriminate between high and low Wee1 expression. Whereas only 20% of the nevi displayed Wee1 expression in ≥10% of the tumor cells, this was the case for 42% of the primary- and 70% of the metastatic tumors. Furthermore, while none of the examined nevi contained >50% Wee1 immunoreactive cells, such expression was found in 4% of the primary melanomas and 22% of the metastatic tissues. Nodular lesions expressed higher levels of Wee1 than the superficial spreading tumors. Wee1 expression was in all cases, except two, exclusively localized to the cell nucleus.

High expression of Wee1 is associated with poor prognosis and increased proliferation

Since expression of Wee1 increased in direction from nevi to primary- and metastatic melanomas, we next examined the relationship between Wee1 expression, clinical parameters and disease outcome. As shown in Table 2, and Figure 1B, high Wee1 expression (in ≥10% of the tumor cells) was significantly associated with thicker primary tumors (p = 0.001), T-staging (p = 0.004), as well as with ulceration (p = 0.005) and poor disease-free survival (p = 0.008). No association with over-all survival was found (data not shown).

Since our panel of melanomas has been previously analyzed for other regulators of the cell cycle, we examined the relationship between Wee1 and expression of these parameters (Ki-67, Cyclin A,- D1,- D3, p21<sup>cip1/Akip1</sup>, p27<sup>kip1</sup> and p53) [11–15]. As shown in Table 2, significant co-variations between Wee1 expression and expression of these parameters were observed (data not shown).

Table 2, significant co-variations between Wee1 expression and expression of these parameters (Ki-67, Cyclin A,- D1,- D3, p21<sup>cip1/Akip1</sup>, p27<sup>kip1</sup> and p53) [11–15]. As shown in Table 2, significant co-variations between Wee1 expression and expression of these parameters were observed (data not shown).

In vitro results support a role of Wee1 in proliferation and genome stabilization

To further study the role of Wee1 in melanomas we knocked-down its expression using siRNA in the three metastatic cell lines, WM239 (p53-wild-type), WM451 (p53-mutated) and LOX (p53-wild-type). Wee1 was effectively silenced in all three cell lines, as confirmed by western blotting; however, phosphorylation on Tyr15 of CDK1, a downstream target of Weel, was only downregulated in WM239 and WM451.1 cells (Figure 2A). Decreased cell viability as estimated by MTS (Figure 2B) and a relative reduction of living cells (Figure 2C), were observed after 24, 48 and 72 hours of siWeel transfection in WM239 and WM451.1, but not in LOX cells.
Furthermore, we observed that Wee1 silencing led to increased cell death in WM 239 and WM 45.1 as determined by the cell death detection ELISA plus kit (Figure 3A). Likewise, cleavage of Poly(ADP-ribose) polymerase (PARP) and pro-caspase-3, markers of apoptosis [19], were detected in the absence of Wee1 (Figure 3B).

Serine 139 phosphorylation of H2AX (γ-H2AX) is a sensitive marker for DNA double-strand breaks, and may be constitutively expressed in untreated cells due to oxidative DNA damage during metabolic activity [20,21]. Expression of γ-H2AX was observed in all cell lines, however in the absence of Wee1, an increase of γ-H2AX was observed in WM239 and WM45.1 cells, indicating increased DNA damage (Figure 3B).

The p53 tumor suppressor protein accumulate in the presence of DNA damages, thus leading to DNA repair, cell cycle arrest or apoptosis [22]. An increase in p53 protein expression was observed in WM239 (p53WT) cells following treatment with siWee1, but not in WM45.1 (p53MUT) or LOX cells (p53WT). Notably, these results indicate that inhibition of Wee1 may sensitize melanoma cell lines to DNA damage regardless of their p53 status.

Since Wee1 is a key regulator of the G2/M phase transition, we studied the effect of Wee1 knockdown on cell cycle progression. As demonstrated in Figure 4A, flow cytometry analysis revealed accumulation of WM239 and WM45.1 cells in the G1/S- and S-phase, respectively. The cell cycle distribution was, however, not affected in siWee1 treated LOX cells. Furthermore, immunoblotting revealed that cyclin D1, -A, and -B1 protein levels were weakly to moderately down-regulated in WM239 and WM45.1, but not in LOX cells. Moreover, a marginal decrease in cyclin D3 expression was observed in WM239 cells. Despite increased p53 expression, p21CIP1/WAF1 protein expression was weakly increased.

Table 1. Number (percentage) of melanocytic lesions expressing different levels of Wee1.

| Expression level | Low (<10%) | 10–50% | >50% |
|------------------|------------|--------|------|
| Nevi             | 0 (0%)     | 8 (80%)| 2 (20%)| 0 (0%)|
| Primary melanoma | 3 (3%)     | 60 (56%)| 41 (38%)| 4 (4%)|
| Superficial spreading | 1 (0%) | 49 (65%)| 24 (32%)| 1 (0%)|
| Nodular          | 2 (6%)     | 11 (33%)| 17 (52%)| 3 (9%)|
| Metastatic       | 1 (4%)     | 6 (26%)| 11 (48%)| 5 (22%)|

Wee1 expressions in benign nevi, primary- and metastatic melanoma were estimated by immunohistochemistry, and categorized in four semi-quantitative classes according to percentage of immunoreactive tumor cells. The groups were further divided into low (<10%) and high (≥10%) expression. doi:10.1371/journal.pone.0038254.t001

Figure 1. High Wee1 expression increases with tumor progression and is associated with a shorter relapse-free period. A. Wee1 expression in cultured melanocytes, benign nevi, primary- and metastatic melanoma, analyzed by immunohistochemistry. B. Melanoma patients were grouped according to Wee1 expression in their tumors (high (n = 44) or low (n = 63)). Relapse-free survival in months was estimated for both groups and presented as a Kaplan Meyer curve.
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in WM239 cells. No alterations were seen in WM45.1 or LOX cells (Figure 4B). As previously reported, p21CIP1/WAF1 was not constitutively expressed in WM45.1 cells, and Wee1 silencing did not affect its expression [23]. The p38 MAP kinase signaling pathway has previously been shown to be involved in p53-independent cell cycle arrest as a response to DNA damage [24], hence we next examined its activation in the absence of Wee1. In support of this hypothesis, increased phosphorylation of p38, indicative of an active signaling pathway, was observed in WM239 and WM45.1, but not in LOX cells, following transfection with siWee1.

Discussion

In the present study, immunohistochemistry was applied to examine the level of Wee1 in a panel of benign nevi and primary – and metastatic melanomas, in order to evaluate the impact of altered expression on disease progression and clinical outcome. We demonstrate that Wee1 up-regulation follows tumor progression and is associated with thicker tumors, ulceration and decreased relapse-free survival. Similar results have previously been reported in other forms of human cancers, such as glioblastoma and breast cancer [5,6]. In non-small-cell lung cancer, on the other hand, reduced Wee1 expression was associated with a higher recurrence rate [8]. Furthermore, Wee1 showed a strong, positive correlation with markers of proliferation: Cyclin A, Ki67 and Cyclin D3 [25]. In support, we have previously reported that increased expression of Ki67, Cyclin A and -D3 is associated with tumor thickness, progression and poor clinical outcome in melanomas [11,12]. In line with these findings, our in vitro results demonstrated that in the absence of Wee1, both Cyclin D1, -D3 (only in WM239) and -A protein expression were weakly decreased in two out of three melanoma cell lines. Based on these findings, we hypothesize that Wee1 contributes to increased proliferation in melanomas.

The augmented expression of Wee1 may seem as a controversy in malignant tumors, based on its well-known inhibitory role in cell cycle progression. However, Wee1 also has a role in genomic stabilization during replication by preventing DNA damage to occur [26,27]. Furthermore, if other mutations have led to increased CDK- activity, elevated levels of Wee1 may be beneficial to avoid premature mitotic entry resulting in cell death [4]. Our in vitro results using siRNA mediated downregulation of Wee1, led to increased cell death, thus further emphasizing the association with malignancy observed in vivo. It is therefore likely that the high
In accordance with a study by Hashimoto et al. [29], using Wee1 inhibitor PD0166585 in murine melanoma, silencing of Wee1 suggested that the p38/MAPK signaling pathway may cause cell cycle arrest after DNA damage in the murine melanomas was even stronger than what was observed in our study following siWee1 transfection. However, whereas siWee1 is believed to be highly specific, PD0166585 is a nonselective Wee1 inhibitor which even at low concentrations can target a range of other kinases involved in regulating CDK activity, such as Membrane-associated tyrosine/threonine protein kinase 1 (MYT1) and Serine/threonine-protein kinase 1 (CHK1) [28,30]. The increased effect may also simply be due to the differences in tumor cell lines. Notably, silencing of Wee1 had no effect on LOX cells in terms of proliferation, cell death or cell cycle distribution. However, phosphorylation of its downstream target CDK1Tyr15 was not abolished in this cell line, thereby providing a possible rationale for lack of response to treatment with siWee1. Hence, we speculate if other mechanisms are more central in CDK1 regulation in this cell line, for instance MYT1, known from other cell systems to have much of the same functions as Wee1 [31].

In the present study we found that Wee1 had a strong positive correlation with p53 expression and p21CIP1/WAF1 in primary melanomas. High p53 expression has previously been shown to correlate with poor clinical outcome and increased proliferation in metastatic melanoma [32,33], however the opposite has also been found [34]. Likewise, we have previously reported that p53 protein expression is increased in metastatic melanoma compared to benign nevi, however, although not significant, high expression was also associated with a more favorable disease progression [15]. Despite being mutated in the majority of human cancers, mutational inactivation of p53 is rare in melanomas; yet the protein may not function as normal. In this regard, it was shown that despite being expressed as wild-type in melanoma, p53 could activate some genes in response to stress, but lacked the ability to inhibit growth or induce apoptosis [33,35]. Interestingly, our in vitro results demonstrated that the effects of silencing Wee1 were not exclusive to p53 mutated cell lines. In contrast to our findings, effects of inhibiting Wee1 in other cancer forms have in previous studies been described as limited to cells with mutated p53, in particular when combined with DNA damaging agents [36–38]. Additionally, p21CIP1/WAF1, a downstream target of p53, was significantly correlated with Wee1 in primary melanomas. p21CIP1/WAF1 is a well-known inhibitor of CDKs, and is known to promote cell-cycle arrest in response to many stimuli, however the protein may also exhibit oncogenic activities [39]. In line with this, we have previously demonstrated that p21CIP1/WAF1 expression is up-regulated in primary melanomas compared to benign nevi, and is associated with thicker tumors [14]. When silencing Wee1 in vitro, p21CIP1/WAF1 expression increased marginally, in WM239 cells only, suggesting that the association between Wee1 and p21CIP1/WAF1 observed in vivo could be due to indirect mechanisms. However, the accumulation of cells in G1/S phase, accompanied by increased p21CIP1/WAF1 protein expression, in siWee1 treated WM239p53mt cells, suggests that the increased p53 protein level probably is able to trigger the G1/S checkpoint in response to DNA damage in this cell line. Hence we speculate if the increased cell death seen in WM239 cells in the absence of Wee1, is related to the cells inability to control CDK activity during DNA replication, rather than the ability to stop the cell cycle progression in G2/M. Notably, Reinhardt et al. has previously reported that activation of the p38/MAPK signaling pathway may cause cell cycle arrest after DNA damage in the absence of p53 [24]. In both WM239 and WM45.1 cells activation of the p38/MAPK signaling pathway increased in the absence of Wee1 suggesting that the p38/MAPK signaling pathway may contribute to the arrest following DNA damages induced by siWee1.
In conclusion, our results indicate that despite being an inhibitor of cell cycle progression, high expression of Wee1 is associated with malignancy and poor prognosis in patients with melanoma. Our in vitro results further support these findings; silencing of Wee1 resulted in DNA damage and increased cell death in two out of three cell lines regardless of p53 status. Thus, high expression of Wee1 appears to protect the cancer cell from DNA damage and ultimately cell death. These findings potentially make Wee1 an eligible target in melanoma, both as mono-therapy and in combination with DNA damaging agents.

Author Contributions
Conceived and designed the experiments: VAF GIM. Performed the experiments: GIM AKRR EE AS. Analyzed the data: GIM RH. Contributed reagents/materials/analysis tools: VAF RH AS. Wrote the paper: GIM VAF.

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