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Mdm2 overexpression and p14^{ARF} inactivation are two mutually exclusive events in primary human lung tumors.

Béatrice Eymin[1, 2], Sylvie Gazzeri[1],[2], Christian Brambilla and Elisabeth Brambilla.

Groupe de Recherche sur le Cancer du Poumon, EA 2021, Equipe INSERM 9924, Institut Albert Bonniot, 38706 La Tronche Cedex, France

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[1.] Both authors contributed equally to this work.

[2] To whom requests for reprints should be addressed.

E-mail: Sylvie.Gazzeri@ujf-grenoble.fr

Beatrice.Eymin@ujf-grenoble.fr

[3] The abbreviations used are: ARF, Alternative Reading Frame; IHC, immunohistochemistry; LCNEC, Large Cell NE Carcinoma; Mdm2, Murine Double Minute 2; NE, Neuroendocrine; NLS, Nuclear Localization Signal; NrLS, Nucleolar Localization Signal; NSCLC, Non-Small Cell Lung Carcinoma; PBS, Phosphate Buffer Saline; PMSF, Phenylmethylsulfonate; pRb, Retinoblastoma protein; SCLC, Small Cell Lung carcinoma; SDS, Sodium Dodecyl Sulfate; TPBS, Tween Phosphate Buffer Saline.

Abstract

Pathways involving *p53* and *pRb* tumor suppressor genes are frequently deregulated during lung carcinogenesis. Through its location at the interface of these pathways, Mdm2 can modulate the function of both *p53* and *pRb* genes. We have examined here the pattern of expression of Mdm2 in a series of 192 human lung carcinomas of all histological types using both immunohistochemical and western blot analyses and four distinct antibodies mapping different epitopes onto the Mdm2 protein. Using Immunohistochemistry (IHC), Mdm2 was overexpressed as compared to normal lung in 31% (60/192) of all tumors analyzed, whatever their histological types. Western blotting was performed on 28 of the 192 tumoral samples. Overexpression of p85/90, p74/76 and p57 Mdm2 isoforms was detected in 18% (5/28), 25% (7/28) and 39% (11/28) of the cases respectively. Overall, overexpression of at least one isoform was observed in 14/28 (50%) lung tumors and concomittant overexpression of at least two isoforms in 7/28 (25%) cases. A good concordance (82%) was observed between immunohistochemical and western blot data. Interestingly, a highly significant inverse relationship was detected between $p14^{ARF}$ loss and Mdm2 overexpression either in NSCLC ($p=0.0089$) or in NE lung tumors ($p<0.0001$). Furthermore, a Mdm2/$p14^{ARF}$ >1 ratio was correlated with a high grade phenotype among NE tumors overexpressing Mdm2 ($p=0.0021$). Taken together, these data strongly suggest that $p14^{ARF}$ and Mdm2 act on common pathway(s) to regulate p53 and/or pRb-dependent or independent functions and that the Mdm2:$p14^{ARF}$ ratio might act as a rheostat in modulating the activity of both proteins.

Introduction

Bronchogenic carcinomas represent the most frequent fatal malignancy in males in Europe and in both sex in United States. They can be divided into different sub-classes

based on the 1999 WHO classification of lung cancer (Travis et al., 1999). These various histological types emerge from pluripotent stem cells that can follow distinct, often overlapping, carcinogenic pathways involving sequential alterations in oncogenes and tumor suppressor genes. *p53* and *pRb* tumor suppressor genes are frequently inactivated during lung tumorigenesis (Brambilla et al., 1993; Brambilla et al., 1999; Chiba et al., 1990; Gazzeri et al., 1994; Gazzeri et al., 1998b; Gouyer et al., 1998; Xu et al., 1996) emphasizing that molecular regulators of these pathways could conceivably be targeted as well.

The Mdm2 protein acts as a bridge over p53 and pRb (Yap et al., 1999). The *Mdm2* gene was initially identified as an amplified gene on a murine double minute chromosome in the spontaneously transformed BALB/c 3T3 cells (Cahilly-Snyder et al., 1987). This oncogenic property was further demonstrated in « *in vitro* » experiments where overexpression of Mdm2 was able to increase the tumorigenic potential (Finlay, 1993) and proliferative rate (Martin et al., 1995) of cultured cells. As well as being a transcriptional target for p53, Mdm2 can also antagonize p53-dependent transcriptional activation and growth arrest by direct binding via its N-terminal region (Chen et al., 1993; Momand et al., 1992; Oliner et al., 1993). Furthermore, Mdm2 can promote the degradation of p53, acting as an ubiquitin-protein ligase to ubiquitinate p53 (Haupt et al., 1997; Kubbutat et al., 1997) and triggering its nuclear export (Geyer et al., 2000; Roth et al., 1998; Tao & Levine, 1999a) and degradation in cytoplasmic proteasomes (Freedman & Levine, 1998; Roth et al., 1998). The inhibitory effect of Mdm2 towards p53 is counteracted by human $p14^{ARF}$, a tumor suppressor gene that acts as a sensor of hyperproliferative signals emanating from oncoproteins (Bates et al., 1998; Zindy et al., 1998). Direct binding of $p14^{ARF}$ to Mdm2 inhibits p53 degradation by blocking p53-Mdm2 nuclear export (Tao & Levine, 1999b; Zhang & Xiong, 1999), sequesters Mdm2 into the nucleolus (Kamijo et al., 1997; Quelle et al., 1995; Weber et al., 1999) and inhibits its ubiquitin

ligase activity (Honda & Yasuda, 1999; Midgley et al., 2000; Pomerantz et al., 1998). By so doing, $p14^{ARF}$ prevents the negative-feedback regulation of p53 by Mdm2 and leads to the activation of p53 in the nucleoplasm. Conversely, high levels of Mdm2 relocalize endogenous $p14^{ARF}$ from nucleoli to nucleoplasm (Zhang & Xiong, 1999) suggesting that balance between both protein levels and their respective subcellular location might be important to regulate their effects.

In addition to its clear role in the regulation of p53, Mdm2 is also able to promote tumorigenesis by interacting with pRb and to inhibit its growth regulatory function through a mechanism(s) actually unknown (Sun et al., 1998; Xiao et al., 1995). In addition, more recent studies have shown that pRb can form a trimeric complex with Mdm2 and p53 and thereby blocks the anti-apoptotic activity of Mdm2 by preventing the degradation of p53 (Hsieh et al., 1999). Thus, depending on the context, interaction between pRb and Mdm2 proteins might mediate distinct effects.

The *Mdm2* gene generates various Mdm2 products, of which only a subset complexes with p53 protein. In addition to the full length protein (p85/90), p54/57 and p74/76 isoforms have been also described as *Mdm2* gene products (Gudas et al., 1995; Haines et al., 1994; Olson et al., 1993). Eventhough the function of these Mdm2 isoforms remains to be elucidated, it is noteworthy that the p74/76 isoforms lacking the N-terminal domain of the full lenght protein do not bind to p53 (Haines et al., 1994; Olson et al., 1993) and can antagonize the ability of p90 to target p53 destruction (Perry et al., 2000). In contrast, the p54/57 isoforms bind to p53 but lack C-terminal epitopes (Olson et al., 1993).

Abnormalities of Mdm2 expression have been reported in human tumors, specially in sarcomas where *Mdm2* gene amplification is commonly observed (Cordon-Cardo et al., 1994; Oliner et al., 1992). In other tumor types, gene amplification is much less common and a variable frequency of Mdm2 overexpression has been described using either immunohistochemical or immunoblot studies (Foulkes et al., 1995; Horie et al.,

2001; Lianes et al., 1994; O'Neill et al., 1998). In human lung tumors, amplification of *Mdm2* gene has been reported in only a few cases of non small cell lung cancer (NSCLC) (Higashiyama et al., 1997; Marchetti et al., 1995) and mutations were never found (Mariatos et al., 2000). In contrast, aberrant expression of Mdm2 product has been variably appreciated since 24 to 70% of NSCLC and 40 to 70% of small cell lung carcinoma (SCLC) were reported to overexpress Mdm2 proteins (Aikawa et al., 2000; Gorgoulis et al., 1996a; Gorgoulis et al., 1998; Gorgoulis et al., 2000; Gorgoulis et al., 1996b; Higashiyama et al., 1997; Stefanaki et al., 1998). Furthermore, a clear overexpression of Mdm2 product has been observed in preneoplastic lung lesions suggesting that alteration of Mdm2 could be an early event during lung carcinogenesis (Rasidakis et al., 1998).

In this study, we investigated the pattern of Mdm2 expression in a large series of human lung cancers of all histological types with known p53, pRb and $p14^{ARF}$ status. By using several antibodies recognizing all Mdm2 isoforms and both immunohistochemical and western blot techniques, we show that Mdm2 is overexpressed in 31% of human lung tumors as compared to normal lung, whatever their histological subtypes. More interestingly, our data demonstrate that Mdm2 overexpression and $p14^{ARF}$ loss are two mutually exclusive events ($p<0.0001$) suggesting that both proteins are located onto common pathway(s) to regulate p53 and/or pRb functions.

Results

Immunohistochemical analysis of Mdm2 protein in human lung tumors.

Mdm2 expression was studied by immunohistochemistry using 4 antibodies mapping distinct epitopes onto the Mdm2 protein (Figure 1A) on a panel of 192 tumor tissue samples of all histological types.

The results were recorded independently by two investigators (EB, CB) who assessed the percentage of positive cells and the intensity of staining. Normal lung parenchyma adjacent to tumor on sections was considered as internal control, whereas 3 normal lung tissues taken for diagnosis in patients without history of cancer were taken as external controls. These tissues contained both positive epithelial and endothelial cells and negative lymphocytes that were the gold standard for comparison in our study. Nuclear staining only was considered to assess Mdm2 immunoreactivity. In normal lung that was present in the vicinity of the tumors as well as in control normal lung tissues, a similar intensity of staining was observed on the entire target population of epithelial and endothelial cells using all antibodies.

Because Mdm2 immunostaining was heterogeneous among lung tumors, differential scores were ascribed in each case and for each antibody according to the intensity of staining and the percentage of stained cells. A mean score was calculated and the resulting data were grouped as described in the methods section into the 4 following classes of staining: 0, undetectable; 1, faint; 2, moderate; and 3, high. According to this, Mdm2 immunostaining in normal alveolar and bronchiolar epithelium and stromal cells (endothelial cells and fibroblasts) was uniformaly distributed between classes 1 and 2 (30 to 80 in extreme score values). Tumors displaying a mean score of class 3 were considered as Mdm2 overexpressing cases since their level of staining was definitely higher than the maximum level reached by normal lung cells. According to this, Mdm2 was overexpressed in 31 of 90 (34%) NSCLC and in 29 out of 102 (28%) NE lung tumors (Table 1 and Figure 2). No significant difference in the pattern of Mdm2 staining was observed between the distinct histological sub-classes of lung tumors. In contrast, Mdm2 overexpression was associated with an extended stage in adenocarcinomas ($p=0.0248$, data not shown).

Possible discordance between antibodies reactivity was investigated according to the localization of their epitope onto the Mdm2 protein. SMP14 and 2A10

antibodies, both recognizing mid-region epitopes in the acidic zinc region of the molecule, always gave a similar score of Mdm2 immunostaining that was lower than those obtained with the two other antibodies in 8% (16/192) of the cases. Absence or abnormal low level of Mdm2 staining was observed in 3% (5/192) and 7% (13/192) of the cases with N20 and C-Ter antibodies respectively, as compared with the high level of reactivity using the 3 others antibodies. Overall, 100% of the tumors analyzed displayed the same pattern of Mdm2 immunostaining (intensity and percentage of positive tumor cells) with at least two antibodies and 88% (168/192) with at least 3 distinct antibodies indicating that a good concordance existed between the antibodies reactivity.

Western blot analysis of Mdm2 in human lung tumors.

In order to validate the IHC data, a western blot analysis was performed onto 28 of the 192 lung tumors using the same four Mdm2 antibodies. The tumors consisted in 6 squamous cell carcinomas, 7 adenocarcinomas, 3 basaloids carcinomas, 4 LCNEC and 8 SCLC and included 7 Mdm2 overexpressing cases based on the IHC analysis.

Using our panel of antibodies and as previously described (Gudas et al., 1995; Haines et al., 1994; Olson et al., 1993), three major Mdm2 isoforms were detected based on their molecular weight of approximately 90kDa (p85/90), 74/76 kDa (p74/76) and 57 kDa (p57/58) (Table 2; Figures 1B & 3). All antibodies reacted with the full-length p85/90 Mdm2 product. Additionally, both SMP14 and C-Ter antibodies recognized the 74/76 isoform whereas N-20 antibody detected the p57 isoform.

The pattern of Mdm2 expression was firstly analyzed in the normal lung. In all samples studied, p85/90 and p57 isoforms were expressed at a relatively low level, whatever the antibody used for p85/90 isoform (Figure 3 & Table 2). A similar low level of p74/76 expression was observed using C-Ter antibody but a higher basal level was found with SMP14 in all these samples suggesting a higher reactivity of SMP14

than that of C-Ter antibody with p74/76 Mdm2 isoform (Table 2). Overall, the pattern of Mdm2 expression in the normal lung using western blotting was consistent with that obtained by IHC.

Also consistent with the IHC data was the wide distribution of Mdm2 expression levels in lung tumors as tumor samples displayed undetectable or low (+), moderate (++), or high (+++) levels of Mdm2 isoforms when compared to normal lung. Of the 28 cases analyzed, 5 (18%; T6, T9, T14, T19, T24) displayed a high level of p85/90 isoform with at least two antibodies (Table 2 & Figure 3). Overexpression of p85/90 was not associated with a specific histological subtype. Discordances of reactivity with one antibody as compared to the others was observed in 11% (3/28) of the cases for C-Ter, 18% (5/28) for N20 and 14% (4/28) for SMP14/2A10 antibodies (Table 2).

A high level of p74/76 isoforms was detected in 7 out of 28 lung tumors (25%) using both SMP14 (1/28, T23) and C-Ter (6/28, T1, T6, T8, T10, T19, T22) antibodies. The lower incidence of p74/76 overexpression using SMP14 is probably linked to its ability to detect a higher level in the normal lung, making difficult the detection of a clear overexpression in tumor samples. Although not statistically significant, overexpression of p74/76 was more frequent in SCLC (3/8, 38%) than in other histological subtype (4/20, 20%).

Finally, p57 isoform was quantitatively more expressed than the two other isoforms as high levels of p57 were observed in 11/28 (39%) lung tumors using N-20 antibody. Interestingly, we noticed that p57 was more frequently overexpressed in SCLC (6/8, 75%; p=0.014) than in other histological sub-types.

Overall, overexpression of all isoforms was observed in 2 cases (T6, T19). p85/90 and p57 were co-overexpressed in 2 cases (T9, T14) and p74/76 and p57 in 3 cases (T1, T8, T10). One case displayed high level of p85/90 only (T24), two others p74/76 only (T22, T23) and four others p57 only (T5, T12, T16, T27). Thus,

overexpression of at least one isoform was observed in 14/28 (50%) lung tumors and overexpression of at least two Mdm2 isoforms was detected in 7/28 (25%) cases. Moreover, we noticed that nearly all the tumors (5/7) with high level of p74/76 co-overexpressed the p57 isoform. Taken together, these data suggest that aberrant expression of several Mdm2 isoforms might occur simultaneously during lung tumorigenesis.

Comparison of IHC and western blot data

Results of western blotting were considered concordant with those of IHC when the same status of Mdm2 was detected with at least two distinct antibodies, whatever the isoform. In these conditions, of the 21 samples considered as Mdm2 non-overexpressing cases using IHC (Table 2), 17 (81%) displayed concordant results by western blotting. In the four other cases (T1, T8, T10, T23), overexpression of Mdm2 was detected using western blot analysis. In two of these samples (T8, T23) however, discordances were observed between the antibodies used in the immunohistochemical analysis. In the last two cases (T1, T10), all antibodies gave concordant IHC data and discordance with western blotting was general.

On the other hand, of the 7 samples considered as Mdm2 overexpressing cases using IHC, 6 (86%, T6, T9, T14, T19, T22, T24) displayed concordant results by western blotting. Interestingly, nearly all these tumors (T6, T9, T14, T19, T22) overexpressed at least two isoforms as detected by western blot analysis. In the last case (T16), overexpression was detected with only one antibody. However, this sample contained more than 50% of stromal cells suggesting that overexpression of Mdm2 in tumor cells was masked by the contamination with low expressing stromal cells. Taken together, these data showed a good concordance (23/28, 82%) between both techniques.

Relationship between Mdm2 and p53 expression in human lung tumors.

The results of Mdm2 immunostaining were compared to those of p53 previously carried out on the same series of tumor samples (Brambilla et al., 1993; Gazzeri et al., 1994). There was no inverse or direct correlation linking Mdm2 and p53 expression in NSCLC and NE lung tumors (Table 3). However, although not statistically significant, we noticed that more than half of the tumors exhibiting Mdm2 overexpression (32/60, 53%), specially adenocarcinomas (11/13, 85%), also displayed a p53 mutant immunophenotype. These results suggest that Mdm2 overexpression can be induced by p53-independent pathway(s).

Relationship between Mdm2 expression and pRb/p16^{INK4a}/cyclin D1 status in human lung tumors.

Alterations of pRb pathway either through pRb loss or p16^{INK4a} loss and/or cyclin D1 overexpression had been previously studied in this series of tumor samples (Brambilla et al., 1999; Gazzeri et al., 1998b; Gouyer et al., 1998) (Table 4). No correlation was found between Mdm2 status and alterations of either pRb pathway or both p53 and pRb pathways in NSCLC or in NE lung tumors (Table 4). However, we noticed that 61% (8/13) of adenocarcinomas exhibited both Mdm2 overexpression and deregulation of p53 and pRb pathways (data not shown).

Comparison of Mdm2 and p14^{ARF} expression in human lung tumors.

Results of p14^{ARF} immunostaining performed previously on the same series of tumor samples (Gazzeri et al., 1998a) have been presented again to allow comparison between the expression of both proteins. Mdm2 overexpression and p14^{ARF} loss were

inversely correlated in NSCLC (p=0.0089) and even more significantly in NE tumors (p<0.0001) (Table 5). Alteration of either Mdm2 or $p14^{ARF}$ expression was never correlated with p53 status. In contrast, it was highly associated with inactivation of pRb pathway in NE lung tumors (p=0.0017; Table 5).

As equilibrium between Mdm2 and $p14^{ARF}$ expression is thought to modulate their activity, Mdm2 and $p14^{ARF}$ scores were compared in all samples analyzed using a paired t test. Although statistically not significant, the mean score of $p14^{ARF}$ was globally higher than that of Mdm2 in all NSCLC and carcinoïds tested (Figure 4A). Conversely, the mean score of Mdm2 was statistically higher than that of $p14^{ARF}$ in LCNEC (p=0.001) and in SCLC (p<0.0001, Figure 4A). Moreover, even when only Mdm2 overexpressing tumors were considered, the mean score of Mdm2 was significantly higher than that of $p14^{ARF}$ in SCLC (p=0.0021) whereas the level of both proteins was not statistically different in all other histological sub-types (Figure 4B).

Discussion

High levels of Mdm2 product are frequently observed in both human cancer cell lines (Gudas et al., 1995) and tumor samples (Foulkes et al., 1995; Gorgoulis et al., 1996a; Gorgoulis et al., 1996b; Horie et al., 2001; Lianes et al., 1994). In most studies, Mdm2 overexpression was assessed by using immunohistochemical studies with one or two antibodies mapping different epitopes onto Mdm2 protein. However, analysis of Mdm2 status is complicated by the existence of various isoforms which detection depends on the antibody used, rendering hazardous the functional interpretation of immunostaining data without any concomittant biochemical analysis. In this study, we analyzed Mdm2 protein status by using both Immunohistochemical and Western blot techniques and four Mdm2 antibodies allowing detection of all Mdm2 isoforms. Because we found a good concordance between the antibodies reactivity in the

immunohistochemical analysis as well as between both IHC and western blot techniques, we are confident that our evaluation of Mdm2 status in our series of human lung tumors is reliable.

We showed a high level of Mdm2 product in 31% of all lung tumors analyzed whatever their histological sub-type. In bronchogenic carcinomas, Mdm2 overexpression was previously reported as a consequence of increased transcription rather than gene amplification (Gorgoulis et al., 1996a; Higashiyama et al., 1997). Beside its constitutively expressed promoter, the *Mdm2* gene contains an additional p53-responsive promoter (Barak et al., 1994). Some previous studies have reported overexpression of *Mdm2* transcript in human tumor cell lines regardless of the endogenous p53 status (Gudas et al., 1995; Ries et al., 2000b). Furthermore, we and others [this study and (Gorgoulis et al., 1996a; Gorgoulis et al., 1998; Gorgoulis et al., 1996b)] showed concomittant Mdm2 overexpression and p53 mutant immunophenotype in lung tumors. Taken together, these data suggest that Mdm2 overexpression can be mediated by p53-independent pathways. A recent study has shown that the *Mdm2* promoter is also a target of the Ras/Raf pathway (Ries et al., 2000a). Accordingly, we and others [this study; (Higashiyama et al., 1997)] observed a slightly more frequent Mdm2 overexpression in lung adenocarcinoma that are known to frequently express a constitutively active Ras mutant (Rodenhuis & Slebos, 1992). Other studies have reported that Mdm2 is also regulated at a post-transcriptional level (Gudas et al., 1995; Khosravi et al., 1999; Maya et al., 2001) as well as by the proteasome (Buschmann et al., 2000; Honda & Yasuda, 1999; Honda & Yasuda, 2000). Overall, these data indicate that several mechanisms might contribute to Mdm2 accumulation during lung tumorigenesis.

According to previous data, we detected three Mdm2 isoforms in lung tissues using western blot analysis. We did not find any correlation between overexpression of either isoform and clinical pathological features, consistent with previous reports

(Gorgoulis et al., 1996b; Ralhan et al., 2000). However, overexpression of p57 isoform was more frequent in SCLC (75%; p=0.0144) as compared to other histological subtypes suggesting its potential implication in the carcinogenesis of high grade NE lung tumors. Interestingly, same predominant overexpression of p57 has been previously described in breast carcinomas (Bueso-Ramos et al., 1996) and chronic lymphocytic leukemias (Haidar et al., 1997). Co-overexpression of at least two isoforms was observed in half of the tumors analyzed indicating that several Mdm2 isoforms can be concomitantly deregulated. The role of the Mdm2 isoforms in tumorigenesis remains unclear. Although p90 and p57 bind to p53 and inhibit its nuclear accumulation (Haines et al., 1994; Olson et al., 1993), p74/76 can stabilize p53 by inhibiting the ability of p90 to stimulate its degradation without affecting the p90/p53 interaction (Perry et al., 2000). Thus, it would be possible that the ratio between the three Mdm2 isoforms determines their effects onto p53 or other cell-cycle regulatory targets.

We did not find any inverse or direct correlation linking Mdm2 and p53 status. However, alteration of p53, as reflected by abnormally stabilized p53 protein, was frequently observed among adenocarcinoma overexpressing Mdm2 protein (11/13, 85%). As overexpression of Mdm2 was statistically associated with extended stages in this histological subtype (this study), our data suggest that alteration of both p53 and Mdm2 expression might contribute to more aggressive features in this subset of NSCLC. Beside its function towards p53, Mdm2 is also able to interact with pRb thereby inactivating its negative growth regulatory function (Sun et al., 1998; Xiao et al., 1995). In high grade NE lung tumors (LCNEC and SCLC), pRb pathway is often inactivated whereas carcinoids exhibit intact pRb pathway. Therefore, Mdm2 overexpression might be a mechanism of pRb impairment in carcinoids whereas both alterations could confer an additive growth advantage for high grade NE lung tumors.

p14^{ARF} and Mdm2 products are functionally closely related. p14^{ARF} acts as an inhibitor of Mdm2 function towards p53 (Honda & Yasuda, 1999; Midgley et al., 2000; Pomerantz et al., 1998; Tao & Levine, 1999b; Zhang & Xiong, 1999). On the other hand, Mdm2 effect(s) onto p14^{ARF} functions remains controversial. Indeed, it was recently shown that loss of Mdm2 could either suppress (Carnero et al., 2000) or restore (Weber et al., 2000) the ability of murine p19^{ARF} to induce p53-independent cell cycle arrest. Thus, depending on the context (cellular types, upstream signals, expression level), Mdm2 could behave either as a mediator or an inhibitor of p14^{ARF} function onto cell proliferation. We found here a striking inverse relationship between Mdm2 and p14^{ARF} status, strongly suggesting that Mdm2 and p14^{ARF} act onto a same pathway, according to the pRb/p16^{INK4a} model. Furthermore, alteration of either Mdm2 or p14^{ARF} expression was significantly associated with inactivation of pRb pathway in high grade NE lung tumors. Altogether, these data suggest that the Mdm2/p14^{ARF} pathway might regulate target genes other than p53 and pRb, such as E2F1 (Eymin et al., 2001). Alternatively, the multiple impairments of the Mdm2/p14^{ARF}/p53/pRb network in these tumors could reflect a "gain of function" phenotype to which the Mdm2/p14^{ARF} balance is the limiting step.

Nucleolar sequestration of Mdm2 by p14^{ARF} leads to p53 accumulation (Kamijo et al., 1997; Quelle et al., 1995; Weber et al., 1999). In contrast, high levels of Mdm2 relocate endogenous p14^{ARF} from nucleoli to nucleoplasm (Zhang & Xiong, 1999). Thus, the balance between both proteins levels might control their subcellular localization and activity. Accordingly, *p14^{ARF}* promoter methylation was recently associated with Mdm2 cytoplasmic localization in human cell lines and tumors (Esteller et al., 2001). In our series of human lung tumors, we did not find any correlation between p14^{ARF} status and Mdm2 subcellular localization (cytoplasmic or nuclear, data not shown). However, we showed that Mdm2 expression levels were significantly higher than those of p14^{ARF} in high grade NE lung tumors (LCNEC,

SCLC) whereas no significant difference was observed in all other histological sub-types. As Mdm2 overexpression can overcome $p19^{ARF}$-mediated growth-arrest (Carnero et al., 2000), our data suggest that imbalance between $p14^{ARF}$ and Mdm2 proteins might contribute to the carcinogenesis of high grade NE tumors. Furthermore, when only Mdm2-overexpressing tumors were considered, Mdm2 level was statistically higher than that of $p14^{ARF}$ only in SCLC, the most aggressive tumors among lung cancers. Thus, it is conceivable that in NSCLC and low grade NE lung tumors (carcinoids) the high level of $p14^{ARF}$ counteracts Mdm2 overexpression, whereas in SCLC the absence or low level of $p14^{ARF}$ predicts its unability to antagonize the oncogenic properties of Mdm2.

In conclusion, we show for the first time a strong inverse relationship between Mdm2 and $p14^{ARF}$ expression in human tumors. These results reinforce the functional link between both proteins and suggest that they act onto same pathway(s) to regulate p53 and/or pRb-dependent or independent functions. A recent study reported that p53 null/Mdm2 heterozygous mice display longer tumor latency, fewer incidence of lymphoma but higher incidence of sarcoma than p53/Mdm2 double null mice (McDonnell et al., 1999). Therefore, depending on the levels of Mdm2 being expressed in the cell and the tissue type, Mdm2 appears to play dual roles *"in vivo"* either as a tumor suppressor or an oncogene. These data endorse the notion of a *Mdm2* gene dosage effect (McDonnell et al., 1999; Weber et al., 2000). According to this, our results suggest that the Mdm2:$p14^{ARF}$ ratio might regulate, as a rheostat, the activity of both proteins thereby modulating the functional consequences of Mdm2 expression. This could explain why overexpression of Mdm2 transcript or protein *"per se"* is not always associated with an adverse prognostic in lung tumors (Higashiyama et al., 1997; Ko et al., 2000).

Material and methods

Tissue samples

One hundred and ninety two lung tumors were included in this study. Tissue samples were taken at surgical resection of lung tumors or at mediastinoscopy of node metastases in order to establish the diagnosis and extension of the disease in non operable patients. Tumor tissues were immediately frozen and kept at -80°C until use. For histological classification, tumor samples were fixed in formalin and/or alcoholic Bouin's fixative. They consisted of 44 squamous carcinomas (42 males, 2 females; 21 stages I/II, 23 stages III/IV), 28 adenocarcinomas (21 males, 7 females; 17 stages I/II, 11 stages III/IV), 18 basaloids carcinomas (18 males, no female; 9 stages I/II, 9 stages III/IV), 26 typical and atypical carcinoids (13 males, 13 females; 24 stages I/II, 2 stages III/IV), 29 Large Cell Neuroendocrine Carcinomas (LCNEC; 27 males, 2 females; 16 stages I/II, 13 stages III/IV) and 47 Small Cell Lung Carcinomas (SCLC; 38 males, 9 females, 4 stages I/II, 43 stages III/IV) according to the 1999 WHO international histological classification of lung tumors (Travis et al., 1999).

Antibodies

Mdm2 protein isoforms were studied using four distinct antibodies mapping different epitopes onto Mdm2. The monoclonal antibodies SMP-14 (Santa Cruz, TEBU, Le Perray en Yvelines) and 2A10 (kindly provided by A. Levine) are directed against residues 154-167 of the acidic domain and 294-339 of the zinc finger domain, respectively. The rabbit polyclonal antibodies N-20 (Santa Cruz, TEBU) and carboxy terminal C-Ter (NCL-Mdm2P, Novocastra, TEBU) recognize residues 1-26 of the amino terminus and an epitope localized near the carboxy terminus of Mdm2 protein, respectively. Antibody to $p14^{ARF}$ (a gift from C. Larsen) has been previously described (Della Valle et al., 1997).

Immunohistochemistry (IHC)

Mdm2 immunostaining was performed on 6 μm thick frozen sections that were previously fixed in 0.4% paraformaldehyde for 10 minutes. Three step immunohistochemical method was applied. Non specific binding sites were blocked by incubating the sections with 0.1% (w/v) bovine serum albumin in phosphate-buffered saline (PBS) for 30 minutes at room temperature. The sections were subsequently incubated with the primary antibody overnight at 4°C using either N-20, C-Ter, SMP14 or 2A10 at 1:4000, 1:2000, 1:500 and 1:2000 dilutions, respectively. Slides were then washed in PBS several times and the primary antibody was detected using biotinylated secondary antibodies consisting of either anti-rabbit biotinylated donkey F(ab')2 (1:1000; The Jackson Laboratory; West Grove; PA) or anti-mouse biotinylated donkey F(ab')2 (1:500; The Jackson Laboratory) for 1 hour at room temperature. Slides were then washed in PBS and incubated with the streptavidin-biotin-peroxydase complex (1:200; Strept-AB complex; DAKO) for 1 hour at room temperature. The chromogenic substrate of peroxydase was a solution of 0.05% 3.3-diaminobenzidine tetrahydrochloride, 0.03% H_2O_2, and 10 mM imidazole in 0.05M Tris buffer (pH 7.6). The slides were counterstained with Harris' hematoxylin. Normal rabbit or mouse IgG at the same concentration as the primary antibodies served as negative controls. Only nuclear staining was considered to assess immunoreactivity. Cytoplasmic staining was never considered in the assessment of Mdm2 expression. The intensity of immunohistochemical staining was evaluated by two independent observers as well as in distinct areas of the slide sections for correlation and confirmation of tissues analysis. Scores of immunostaining were calculated by multiplying the percentage of labeled cells with the intensity of staining (1+, 2+, 3+) and tumors were graded into four classes based on their score (class 0: no staining; class 1: < 50; class 2: 50-100; class 3 ≥ 100). Tumors with more than 50% of tumor cells displaying moderate or strong nuclear staining were considered as tumors

overexpressing Mdm2 (score ≥ 100; class 3) when compared to normal tissues. p53, pRb and $p14^{ARF}$ immunostaining have been previously described (Brambilla et al., 1993; Brambilla et al., 1999; Gazzeri et al., 1998a ; Gouyer et al., 1998).

Western-blot analysis

Fifteen normal lung tissues and 28 representative tumor samples of the IHC series were analyzed using western blotting. These samples were taken at the immediate vicinity of those studied by immunohistochemistry. Tissues were lysed in ice-cold lysis buffer (5 mM EDTA, 150 mM NaCl, 100mM Tris (pH 8), 0.5% sodium deoxycholate, 0.5% nonidet 40, 0.5% sodium dodecyl sulfate (SDS), 0.1% aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 mM PMSF) for 30 min and centrifugated for 30 min at 15000g. Supernatants were then collected and frozen at –80°C until use. Proteins (40 μg) were denatured in Laemmli buffer (60 mM Tris HCl (pH 6.8), 20% glycerol, 10% ß-mercaptoethanol, 4.6% SDS, and 0.003% bromophenol blue), separated by 7-10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electroblotted on PVDF membrane (Hybond P, Amersham, Les Ulis, France). The membrane was then incubated for 2-3 hours at room temperature with primary antibody in 2% non-fat milk TPBS, washed three times in TPBS, incubated with secondary horseradish peroxydase-conjugated goat anti-mouse or anti-rabbit antibodies (The Jackson Laboratory, West Grove, PA) for 30 minutes and revealed using enhanced chemoluminescence detection kit (ECL; Amersham). Dilutions of the primary antibodies were 1:10.000, 1:20.000, 1:20.000 and 1:5000 for 2A10, N-20, SMP14 and C-Ter respectively. To ensure equal loading and transfer of proteins, the membranes were subsequently probed with a polyclonal actin antibody (1:500; Sigma-Aldrich; L'Isle d'Abeau).

Statistical Analyses

All statistical correlations were based on the chi-square (X^2) test. Additional Student paired-T test was performed as mentionned in the text.

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Legends to figures

Figure 1. Reactivity of Mdm2 antibodies onto Mdm2 protein.

A, Major functional domains of Mdm2 protein. Binding sites of some Mdm2 targets p53, pRb and $p14^{ARF}$ are shown at the top. The hatched boxes indicate nuclear localization (NLS), export (NES) and nucleolar localization signals (NrLS) as mentionned. The black boxes indicate zinc fingers. The acidic domain and the RING finger are also represented. At the bottom, reactivity of N20, SMP14, 2A10 and C-Ter antibodies with the full length Mdm2 protein epitopes is indicated. B, Representation of the three major Mdm2 isoforms, p85/90, p74/76 and p57/58. p85/90 contain all Mdm2 epitopes, p74/76 is devoided of N-terminal epitope that maps between residues 19 to 50 (region required for binding to p53) and p57 has deletion of the C-terminal epitope.

Figure 2. Immunohistochemical analysis of Mdm2 in normal lung and lung tumors.

A, Mdm2 nuclear immunostaining with SMP14 antibody in normal bronchial basal and suprabasal cells (score 50) (x200); B, Normal lung alveolar parenchyma stained with N20 antibody. Type II cells show moderate nuclear staining (arrow) (score 80) (x200); C, Squamous cell carcinoma immunostained with C-Ter antibody showing a faint Mdm2 nuclear expression (score 20) (x200). Note the moderate Mdm2 expression in entrapped type II cells (arrow); D, Squamous cell carcinoma exhibiting a strong nuclear staining with 2A10 antibody (score 150) (x200); E, Small cell lung carcinoma immunostained with N20 antibody (score 50) (x200); F, Large cell neuroendocrine carcinoma immunostained with SMP14 antibody (score 180) (x200); G-H, Small cell lung carcinoma showing discordances between a high nuclear staining

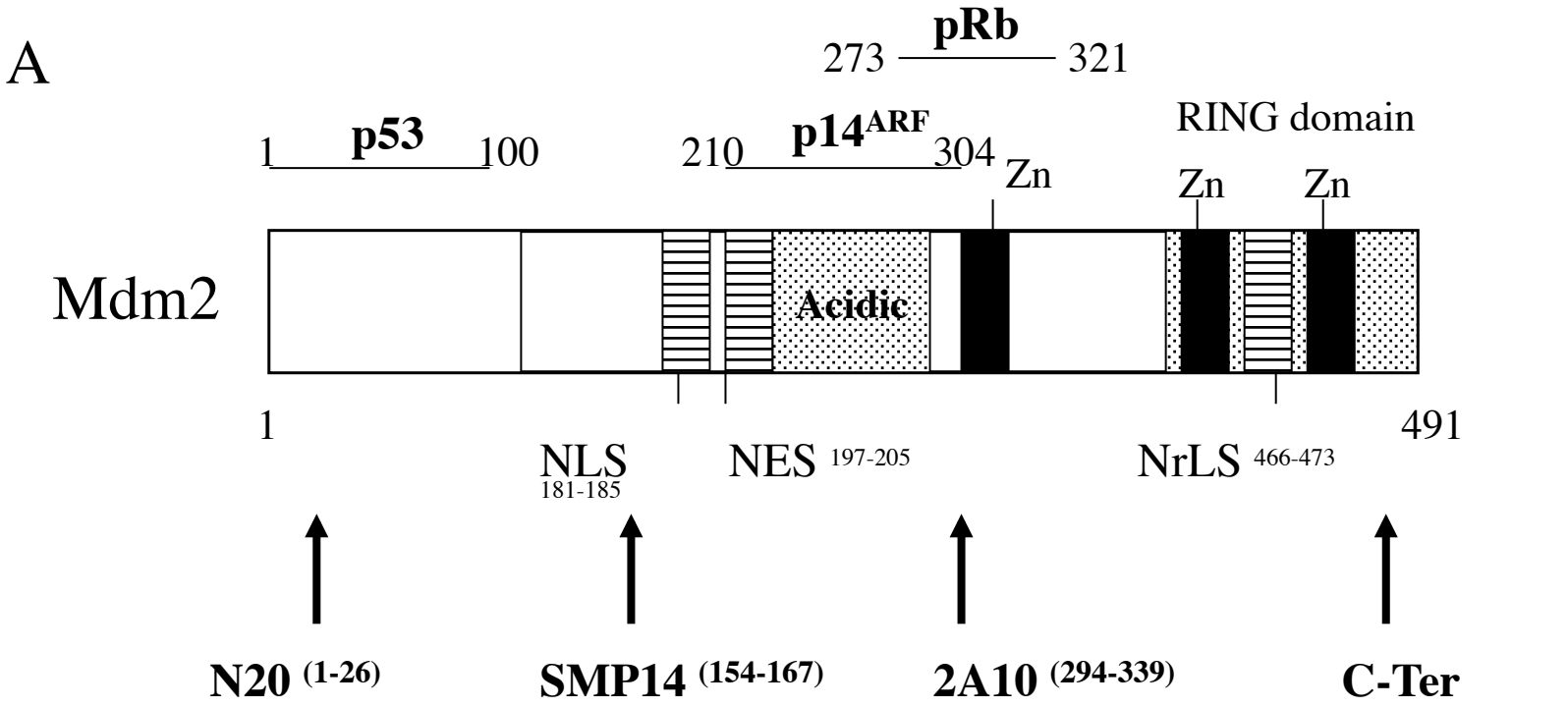
with N20 antibody (score 100) and a low nuclear staining with C-Ter antibody (score 30) (x200) leading to a final moderate mean score (score 65).

Figure 3. Representative western blots showing the expression of Mdm2 isoforms in lung tumors.

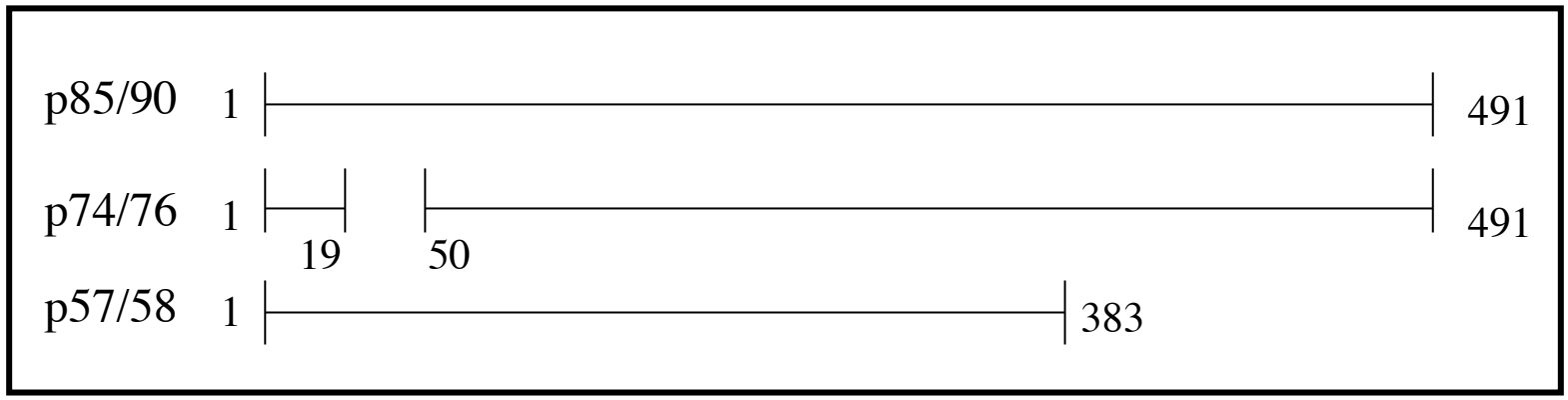
Expression of Mdm2 isoforms in tumor samples (T) was compared to that of normal lung (NL). A, Immunoblotting with 2A10 antibody. T6 and T19 are representative of lung tumors that overexpress p85/90 isoform. B, Immunoblotting with N20 antibody. T6 overexpress both p85/90 and p57 isoforms and T16 p57 isoform only. C, Immunoblotting with SMP14 antibody. Overexpression of p85/90 in T6 sample according to 2A10 and N20 staining. T23 is the only tumor that overexpress p74/76 isoform using SMP14. D, Immunoblotting with C-ter antibody. T8 and T22 are representative of tumors that overexpress p74/76 isoform. T24 exhibits high levels of p85/90 only.

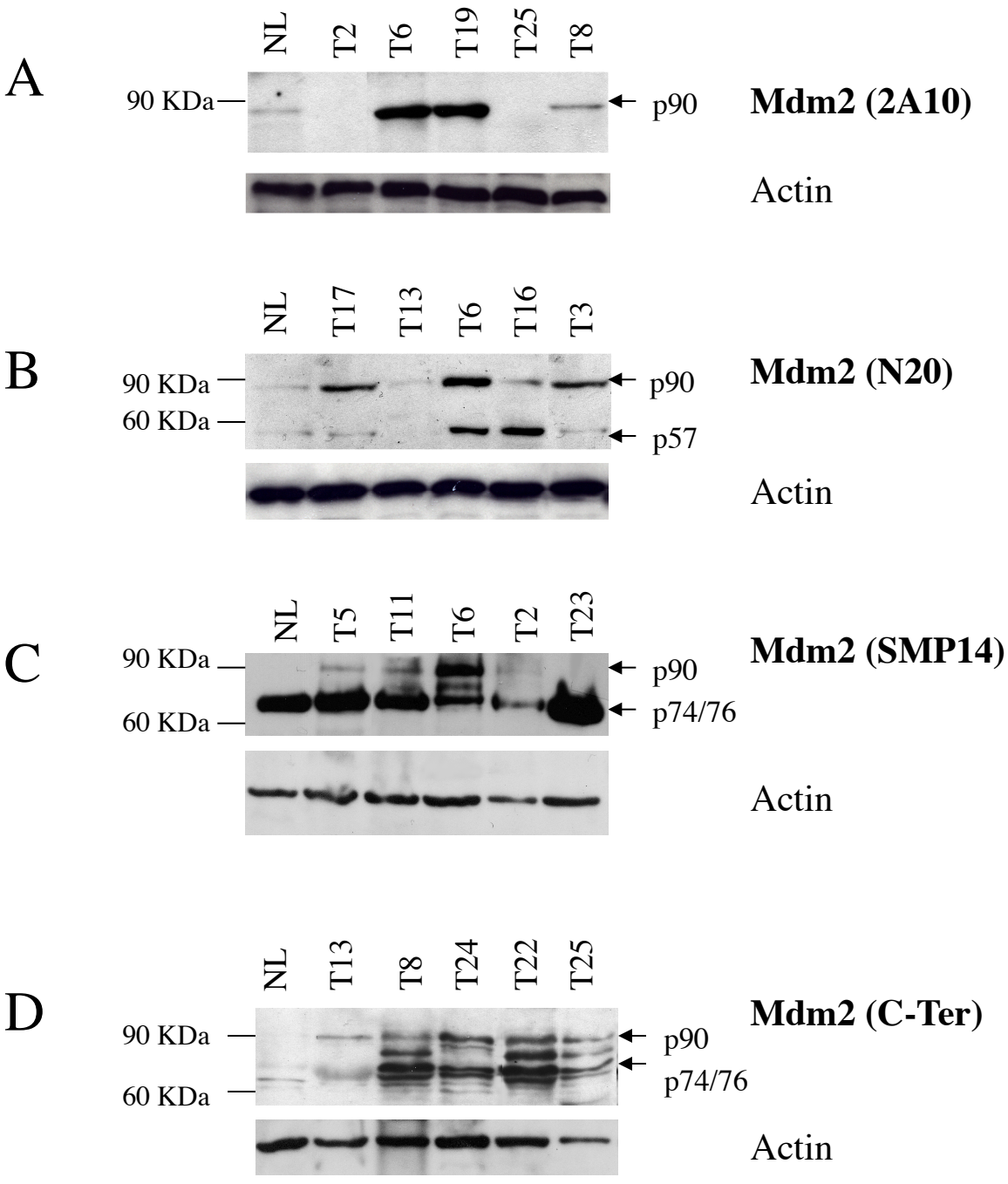
Figure 4. Comparison of Mdm2 and $p14^{ARF}$ mean scores according to the histological type of human lung tumors.

Mdm2 and $p14^{ARF}$ scores were evaluated in each tumor sample using immunohistochemistry. Mean scores were calculated for each histological sub-type of lung tumors and compared using a paired t-test. Mdm2 and $p14^{ARF}$ mean scores are presented ± SD (standard deviation) and compared in all samples (A) and in samples overexpressing Mdm2 protein (B). SCC, Squamous Cell Carcinoma; ADK, Adenocarcinoma; LCNEC, Large Cell Neuroendocrine Carcinoma; SCLC, Small Cell Lung Carcinoma; NS, non significant.


A
pRb
273
321
p53
1
100
p14ARF
210
304
RING domain
Zn
Zn
Zn
Mdm2
Acidic
1
491
NLS
181-185
NES 197-205
NrLS 466-473
N20 (1-26)
SMP14 (154-167)
2A10 (294-339)
C-Ter

B


p85/90
1
491
p74/76
1
491
19
50
p57/58
1
383


A
NL
T2
T6
T19
T25
T8
90 KDa
p90
Mdm2 (2A10)
Actin
B
NL
T17
T13
T6
T16
T3
90 KDa
60 KDa
p90
p57
Mdm2 (N20)
Actin
C
NL
T5
T11
T6
T2
T23
90 KDa
60 KDa
p90
p74/76
Mdm2 (SMP14)
Actin
D
NL
T13
T8
T24
T22
T25
90 KDa
60 KDa
p90
p74/76
Mdm2 (C-Ter)
Actin

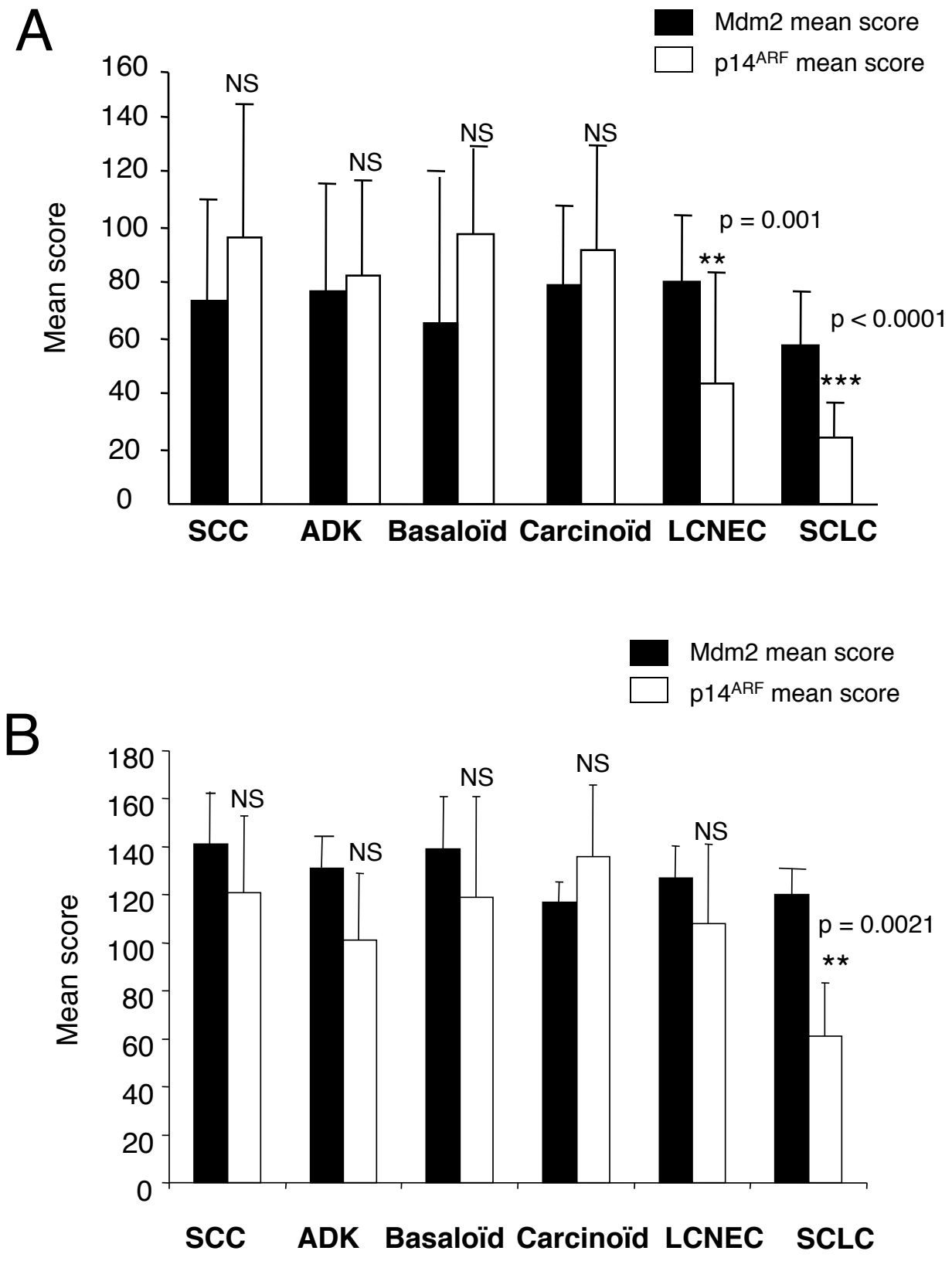

A
Mdm2 mean score
p14ARF mean score
Mean score
160
140
120
100
80
60
40
20
0
NS
NS
NS
NS
p = 0.001
**
p < 0.0001
***
SCC
ADK
Basaloïd
Carcinoïd
LCNEC
SCLC
B
Mdm2 mean score
p14ARF mean score
Mean score
180
160
140
120
100
80
60
40
20
0
NS
NS
NS
NS
NS
p = 0.0021
**
SCC
ADK
Basaloïd
Carcinoïd
LCNEC
SCLC

Table 1: Immunohistochemical analysis of Mdm2 in human lung tumors.

Histological types	n	Mdm2 expression (%) [a]				p^{d}	Negative epitopes (%) [b]		
		0	1	2	3		**N** N20	**Ac Z** 2A10, SMP14	**3C** C-Ter
Squamous cell carcinoma	44	2	15	13	14		3	6	4
Adenocarcinoma	28	0	12	3	13		0	3	2
Basaloid	18	0	9	5	4		0	3	2
Total NSCLC	**90**	2 (2)	36 (40)	21 (24)	**31 (34)**	NS	**3**	**12**	**8**
Carcinoid [c]	26	0	6	12	8		0	0	0
LCNEC	29	2	4	12	11		0	0	1
SCLC	47	1	20	16	10		2	4	4
Total NE lung tumors	**102**	3 (3)	30 (29)	40	**29 (28)**	NS	**2**	**4**	**5**
Total Tumors	**192**	5 (3)	66 (34)	61 (32)	**60 (31)**		**5 (3)**	**16 (8)**	**13 (7)**

[a] A score of Mdm2 staining was attributed for each antibody to each sample analyzed by multiplying the percentage of labelled cells (0-100%) by the intensity of staining. A mean score was then calculated and the resulting data were grouped into the 4 following classes of expression: no stained cells, 0; x <50, class1; 50< x <100, class 2; x ≥100, class 3.

[b] Negative epitopes means number of tumors exhibiting absence or abnormal low level of mdm2 staining with the antibody corresponding to: N-terminal (N20 20 N-terminal aminoacids), acidic zinc region (2A10 and SMP14) and carboxyterminal (C-ter) regions as compared with reactivities of other epitopes.

[c] Carcinoid includes typical and atypical subtypes.

[d] χ^2 test. NS, non significant.

LCNEC, Large Cell Neuroendocrine Carcinoma; SCLC, Small Cell Lung Carcinoma.

Table 2: Expression of Mdm2 isoforms using western blot analysis and concordance with IHC data.

Patients	IHC [a] (0 to 3)	Western blot analysis [b] 2A10 p85/90	N-20 p85/90	N-20 p57	SMP14 p85/90	SMP14 p74/76	C-Ter p85/90	C-Ter p74/76
Normal lung	1-2	+	+	+	+	++	+	+
LCNEC								
T1	1	++	+	+++	+++	+	+	+++
T2	1	+	+	+	+	+	+	+
T3	2	+	++	+	+	++	++	+
T4	1	+	++	+	+	+	ND	ND
SCLC								
T5	1	++	++	+++	++	+	++	++
T6	**3**	**+++**	**+++**	**+++**	**+++**	**+**	**+**	**+++**
T7	1	+	++	+	++	+	+	+
T8	2	++	++	+++	++	+	+	+++
T9	**3**	**+++**	**+**	**+++**	**+++**	**++**	**++**	**++**
T10	1	+++	+	+++	+	+	+	+++
T11	2	+	++	+	++	+	+	+
T12	1	++	+	+++	+	+	ND	ND
ADK								
T13	2	+	+	+	+	+	+	+
T14	**3**	**+++**	**++**	**+++**	**+++**	**++**	**ND**	**ND**
T15	1	+	++	+	+	++	+	++
T16	**3**	**++**	**++**	**+++**	**++**	**+**	**+**	**++**
T17	1	+	++	+	+	+	+	+
T18	1	++	+	+	++	+	+	+
T19	**3**	**+++**	**++**	**+++**	**+++**	**++**	**+**	**+++**
SCC								
T20	2	+	+	+	+	+	++	++
T21	2	++	++	+	+	+	+	+
T22	**3**	**+**	**+**	**+**	**+++**	**++**	**++**	**+++**
T23	2	+	+++	+	+	+++	++	++
T24	**3**	**+**	**+**	**+**	**+++**	**+**	**+++**	**++**
T25	1	+	+	+	+	+	+	+
Basaloid								
T26	1	++	++	++	+	+	++	+
T27	2	++	+	+++	++	++	+	+
T28	1	++	++	++	++	++	+	++

[a] IHC, immunohistochemistry.
Differential classes of Mdm2 expression were attributed to each sample as described in the material and methods section.

[b] Western blot analysis of Mdm2 isoforms using distinct antibodies. ND : not done.
Expression of each isoform was classified as: +, undetectable or low level; ++, moderate level; +++, high level.
LCNEC, Large Cell Neuroendocrine Carcinoma; SCLC, Small Cell lung Carcinoma; ADK, Lung Adenocarcinoma;
SCC, Squamous Cell Carcinoma.
Cases with Mdm2 overexpression are indicated in high solid.

Table 3: Relationship between Mdm2 and p53 immunohistochemical status in human lung tumors.

Histological class	n	Mdm2 IHC classes 0 to 2 [a]		Mdm2 IHC class 3 [b]		*P*[a]
		p53 WT[c]	p53 MT[d]	p53 WT[c]	p53 MT[d]	
Squamous cell carcinom	44	5	25	6	8	
Adenocarcinoma	28	6	9	2	**11**	
Basaloid	18	0	14	2	2	
Total NSCLC	**90**	11	48	10	**21**	NS
Carcinoid	26	17	1	7	1	
LCNEC	29	4	14	6	5	
SCLC	47	16	21	5	5	
Total NE lung tumors	**102**	37	36	18	11	NS
Total Tumors	**192**	**48**	**84**	**28**	**32**	NS

[a] Classes of Mdm2 expression corresponding to a mean score <100 using immunohistochemistry.
[b] Class 3 of Mdm2 expression corresponding to a mean score ≥100 and considered as Mdm2 overexpressing cases.
[c] p53 WT: wild-type p53 as reflected by negative immunostaining.
[d] p53 MT: mutant immunophenotype of p53 as reflected by positive immunostaining.

Table 4: Relationship between Mdm2 status and pRb pathway in human lung tumors.

Histological class	n	Mdm2 IHC classes 0 to 2 [a]		Mdm2 IHC class 3 [b]		*P*[e]
		pRB WT[c]	pRB MT[d]	pRB WT[c]	pRB MT[d]	
Squamous cell carcinoma	44	7	23	3	11	
Adenocarcinoma	28	4	11	4	9	
Basaloid	18	1	13	1	3	
Total NSCLC	**90**	12	47	8	23	NS
Carcinoid	26	13	5	8	0	
LCNEC	29	1	17	3	8	
SCLC	47	3	34	0	10	
Total NE lung tumors	**102**	17	56	11	18	NS

[a] Classes of Mdm2 expression corresponding to a mean score <100 using immunohistochemistry.
[b] Class 3 of Mdm2 expression corresponding to a mean score ≥100 and considered as Mdm2 overexpressing cases.
[c] pRB WT means that pRb pathway is not inactivated either through pRb loss, $p16^{INK4a}$ loss or cyclin D1 overexpression.
[d] pRB MT means that either RB or $p16^{INK4a}$ proteins are lost and/or that cyclin D1 is overexpressed.
[e] X^2 test. NS, non significant.

Table 5: Relationship between Mdm2 and $p14^{ARF}$ expression in human lung tumors. Correlation of Mdm2/$p14^{ARF}$ status with pRb pathway.

Histological class	n	Mdm2 IHC classes 0 to 2 [a]		Mdm2 IHC class 3 [b]		P[d]	$p14^{ARF}$ + [c] or Mdm2 <100 [a]		$p14^{ARF}$ - [c] or Mdm2 ≥100 [b]		P[d]
		$p14^{ARF}$ + [c]	$p14^{ARF}$ - [c]	$p14^{ARF}$ + [c]	$p14^{ARF}$ - [c]		pRbWT [e]	pRb MT [e]	pRbWT [e]	pRb MT [e]	
Squamous cell carcinoma	44	22	8	14	0		6	16	4	18	
Adenocarcinoma	28	11	4	12	1		4	7	4	13	
Basaloid	18	11	3	4	0		1	10	1	6	
Total NSCLC	**90**	**44**	**15**	**30**	**1**	P= 0.0089	**11**	**33**	**9**	**37**	NS
Carcinoid	26	15	3	8	0		12	3	9	2	
LCNEC	29	5	13	9	2		0	5	5	19	
SCLC	47	8	29	8	2		2	6	0	39	
Total NE lung tumors	**102**	**28**	**45**	**25**	**4**	P < 0.0001	**14**	**14**	**14**	**60**	0.0017

[a] Classes of Mdm2 expression corresponding to a mean score <100 using immunohistochemistry.

[b] Class 3 of Mdm2 expression corresponding to a mean score ≥100 and considered as Mdm2 overexpressing cases.

[c] $p14^{ARF}$ +, positive immunostaining reflecting expression of $p14^{ARF}$ protein;
$p14^{ARF}$ -, negative immunostaining reflecting loss of $p14^{ARF}$ protein.

[d] X^2 test. NS, non significant.

[e] pRB WT means that pRb pathway is not inactivated either through pRb loss, $p16^{INK4a}$ loss or cyclin D1 overexpression.
pRB MT means that either RB or $p16^{INK4a}$ proteins are lost and/or that cyclin D1 is overexpressed.