Identification of tumour-suppressor-gene candidates in small cell lung cancer, which alone or combined with a p53-reactivating molecule, can be used for cancer gene therapy.

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Background

- Small cell lung cancer (SCLC) is a deadly disease with no current satisfactory treatments. It is a highly metastatic disease and is generally disseminated at the time of diagnosis.
- There is therefore an urgent need for development of more efficient treatment modalities for patients with SCLC.
- One intriguing modality is tumour-suppressor-restoration-therapy.
- As different types of cancers have different tumour suppressor deficiencies, the strategy must be customized to each cancer type, either by using tumour specific tumour suppressor genes (TSG) or by combining different TSG.
- p53 is mutated in up to 90% of SCLC tumours, and as mutant p53 tend to accumulate in tumour cells, an effective approach would be to reactivate mutant p53 in cancer cells combined with reintroduction of another TSG, frequently inactivated in SCLC.
- PRIMA-1^{MET} (p53-dependent reactivation and induction of massive apoptosis) is a small molecule reported to have the capability of restoring tumour-suppressor function to mutant p53 in various cancer cell lines and hence induce cancer cell death.
- Whether PRIMA-1^{MET} is capable of reactivating mutant p53 in SCLC cells in not yet known.

Aim

- To test the effect of PRIMA-1^{MET} on SCLC cell lines with mutant p53.
- To identify TSG candidates in SCLC, which alone or combined with PRIMA-1^{MET} can be tested for use in SCLC gene therapy.

Materials and Methods

- **Detection of p53 mutations**: RNA was extracted from various SCLC cell lines and p53 cDNA was synthesized using RT-PCR and sequenced. The results were compared to a sequenced 1285 nucleotide fragment covering the whole coding region of wt-p53. This study was performed by *Abrahamsen N, Petri A, Jensen TV, Thomsen MS og Poulsen HS*.
- **Western Blot analysis**: Cells were lysed in RIPA lysis buffer and equal amounts of protein resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes. Proteins of interest were detected by immunoblotting in an Autochemi System (UVP).
- **MTT assay**: Cells were seeded in 96-well plates and incubated with various concentrations of PRIMA-1^{MET} for 72 h. The plates were incubated for 4h with MTT, after which a solubilization buffer (10% SDS, 0,03 M HCl) was added.
- **Transient transfection**: Cells were transiently transfected with expression vectors encoding either Cyr61 or WISP2 using Lipofectamine 2000.

Results

- The mutation status of p53 in a panel of SCLC cell lines was previously evaluted in our Laboratory (**Table 1).**
- In some of the SCLC cell lines, high levels of mutant p53 was detected (**Figure 1**).
- PRIMA-1^{MET}, a methylated form of the original compound PRIMA-1 (**Figure 2**), inhibited the growth of the SCLC cell lines with high levels of mutant p53 markedley, but only caused a minor reduction in growth rate in the control cell lines H1299 (p53 null) and CCD32Lu (wt-p53).
- The IC50 values for PRIMA-1^{MET} varied depending on cell lines tested. However, SCLC cell lines expressing mutant p53 had a lower IC50 value than those with wt-p53 (CCD32Lu) or p53 null (H1299) (**table 2**).
- Unlike H1299, PRIMA-1^{MET} treatment of the SCLC cell lines DMS273, GLC16 induced the expression of the two p53 target genes p21 and MDM2 and resulted in caspase-3 activation, PARP cleavage, and decrease in total levels of mutant p53 (**Figure 4**). This was also observed in the GLC14 and GLC19 cell lines (data not shown)
- Cyr61 and WISP2 are two genes the expression of which is markedley dowregulated in SCLC cell lines compared to normal cells and tissues (**Figure 5**).
- Cyr61 and WISP2, who have previously been reported to act as TSG[2,3], were tested for their tumor suppressor activity in SCLC cell lines by transient transfection (**Figure 6**).
- Neither of the genes had a cytotoxic effect in the cell lines tested (**Figure 6**).

Conclusions

- PRIMA-1^{MET} seems to reactivate mutant p53 in SCLC, leading to transactivation of target genes, and subsequently apoptosis.
- Cyr61 and WISP do not seem to be effective alone but might induce pronounced growth suppression when combined with PRIMA-1^{MET}

1. Bykov V *et. al.*, 2002. Restoration of the tumor suppressor function to mutant p53 by low molecular-weight compond, Nature Medicine, 8: 282-288.
2. Pennica, D., *et el.* 1998. WISP genes are members of the connective tissue growthfactor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors.Natl. Acad. Sci. U.S.A.95:14717-14722.
3. Xiangjun, T., *et al.* 2001. Cyr61, a member of CCN family, is a tumor suppressor in non-small cell lung cancer.J Biol Chem, 276(50): 47709-14.

Cell line	allel	mutation	Amino acid change	domain	Transfection effeciency
CPH 54A	215G/B,72R/P (WT)	ligat			high
CPH 54B	215 G/B,72R/P (WT)	ligat			high
GLC2	215 G; 72R (WT)	delta700-000	delta 204-200	C	high
GLC 3	215 G; 72R (WT)	G150A	W50UGA (STOP)	A-E	low
GLC 14	215 G; 72R (WT)	G010T	R273L	C	high
GLC 16	215 G; 72R (WT)	G010T	R273L	C	high
GLC 19	215 G; 72R (WT)	G818T	R273L	C	medium
GLC 26	215 G; 72R (WT)	C844T	R282W	C	medium
GLC 28	215 G; 72R (WT)	C844T	R282W	C	medium
DMS 53	215 G; 72R (WT)	G711T	S237F	C	medium
DMS 79	215 G; 72R (WT)	1 G826A	G276/Stopframeshift	C-E	medium
DMS 92	215 G; 72R (WT)	G711A	M237I	C	medium
DMS 114	215 G; 72R (WT)	C637T	R213U GA(STOP)	C-E	medium
DMS 153	215 G; 72R (WT)	A163C	T155P	C	low
DMS 273	215 G; 72R (WT)	G818T	R273L	C	high
DMS 454	215 G; 72R (WT)	A536T	H179L	C	low
DMS 79	215 G; 72R (WT)	G44RT	V15TF	C	low
NCI H69	215 G, 72R (WT)	G011T	E171erUes	C-E	high
NCI 417	215 C, 72P (WT)	G838T	G331H	D	medium
Mel 24H	215 C; 72P (WT)	C832G	P278A	C	medium
Mar 86t	215 C; 72P (WT)	delta 96 114	L32frameshift	A E	low

Table 1. p53 mutation status in various SCLC cell lines was determined as described in Materials and Methods and performed by *Niels Abrahamsen, Andreas Petri, Tine V. Jensen, Mogens S. Thomsen and Hans S. Poulsen . The p53 protein is 393 amino acids (aa) long and consists of 5 domains referred to as domain A-E. Domain A (aa 1-62): transactivation domain; B (aa 63-94): proline-rich domain; C (aa 95-292): DNA binding domain; D (aa 325-356): tetramerization domain; E (aa 357-393): negative auto-regulatory domain. Transfection efficiency was determined by reporter gene expression.*

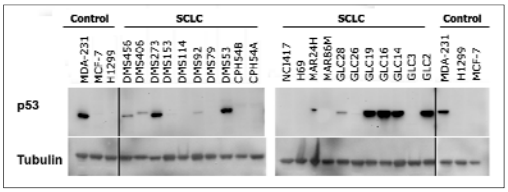

Figure 1. Total protein levels of p53 was evaluated in the indicated cell lines by Western Blot analysis. The human breast cancer cell lines MDA-231 and MCF-7, which express endogenous mutant and wild type p53, respectively, and the human non-small cell lung carcinoma (NSCLC), H1299, which is p53 null, were used as control. Tubulin was used for control of protein loading.

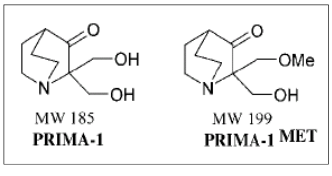

Figure 2. Structural formula of PRIMA-1 and a methylated form of this compound, PRIMA-1^{MET}, which has been shown to be more active than the original. Figure from Bykov et. al.[1]

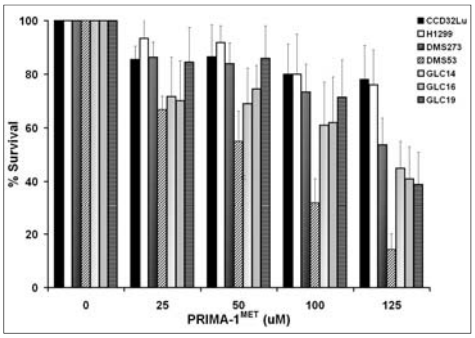

Figure 3. The growth suppression effect of different concentrations of PRIMA-1^{MET} on a panel of SCLC cell lines with p53 mutation (DMS53, DMS273, GLC14, GLC16 and GLC19) was studied using MTT assay. The non-transformed diploid human lung fibroblast cell line, CCD32Lu, expressing wild type p53 and the p53 null NSCLC cell line H1299, were used as control cell lines.

Table 2.

Cell Line	IC50 (uM)
DMS53	48
DMS273	125
GLC14	110
GLC16	114
GLC19	105
CCD32Lu	140
H1299	130

Table 2. The IC50 (concentration of a drug that causes 50% growth inhibition) values for PRIMA-1^{MET} in the indicated cell lines. Cells were plated in 96-well plates and varoius concentrations of PRIMA-1^{MET} ranging from 0-400 uM were added and incubated for 72 hours.

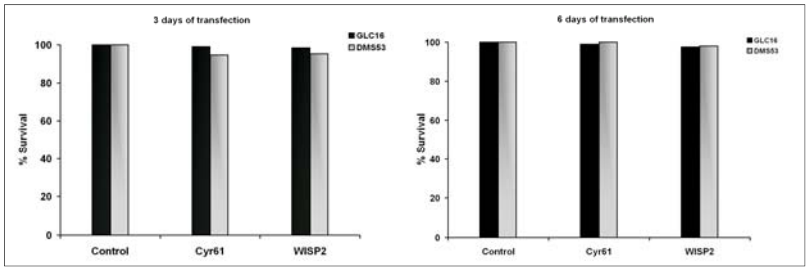

Figure 6. The GLC16 and DMS53 cells lines were transiently transfected with expression vectors encoding either Cyr61 or WISP2. The cells were also transfected with an empty vector (control). The tranfected cells were plated in 96-well plates and the effect of gene expression on growth suppression was evaluated by MTT assay after 3 or 6 days of transfection.

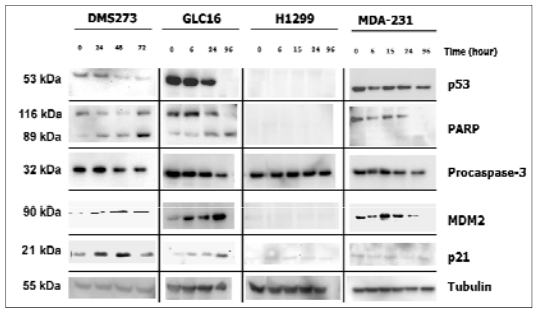

Figure 4. Total levels of p53, PARP, Procaspase-3, MDM2, p21 and tubulin (control for protein loading) was evaluated in the indicated cells lines after treatment with 100 uM PRIMA-1^{MET} for the indicated time periods by Western blot analysis. Equal amounts of protein (30 or 50 ug) protein was loaded in each lane and the proteins were visualized by sequential immunoblotting and stripping. The H1299 and MDA-231 cell lines were included as controls.

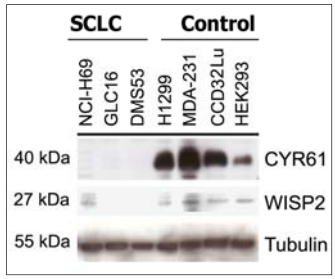

Figure 5. The protein levels of Cyr61 and WISP2 were evaluated in the indicated cell lines. Equal amounts of protein (10 ug) was loaded in each lane and Cyr61, WISP2 and Tubulin were visualized by sequential immunoblotting and stripping. H1299, MDA-231, CCD32Lu and the human embryonal kidney epithelial cell line, HEK293, were used as controls as the level of Cyr61 and WISP2 in these cell lines has been reported to be high.