The role of

Alternative Lengthening of Telomeres

in human cancer

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Statement of Originality

The contents of this thesis have not been presented for the award of a degree or diploma at this or any other university. The data presented are the original work of the author except where specifically indicated in the text.

Jeremy D. Henson

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Publications

1. Henson,J.D., Neumann,A.A., Yeager,T.R., and Reddel,R.R. (2002). Alternative lengthening of telomeres in mammalian cells. Oncogene 21, 598-610.

2. Henson,J.D., Hannay,J.A., McCarthy,S.W., Royds,J.A., Yeager,T.R., Robinson,R.A., Wharton,S.B., Jellinek,D.A., Arbuckle,S.M., Yoo,J., Robinson,B.G., Learoyd,D.L., Stalley,P.D., Bonar,S.F., Yu,D., Pollock,R.E., and Reddel,R.R. (2005). A robust assay for alternative lengthening of telomeres (ALT) in tumors demonstrates the significance of ALT in sarcomas and astrocytomas. Clin. Cancer Res. 11, 217-225.

3. Jeyapalan,J.N., Varley,H., Foxon,J.L., Pollock,R.E., Jeffreys,A.J., Henson,J.D., Reddel,R.R., and Royle,N.J. (2005). Activation of the ALT pathway for telomere maintenance can affect other sequences in the human genome. Hum. Mol. Genet. 14, 1785-1794.

4. Jiang,W.Q., Zhong,Z.H., Henson,J.D., Neumann,A.A., Chang,A.C.M., and Reddel,R.R. (2005). Suppression of alternative lengthening of telomeres by Sp100-mediated sequestration of MRE11/RAD50/NBS1 complex. Mol. Cell. Biol. 25, 2708-2721.

5. Costa,A., Daidone,M.G., Daprai,L., Villa,R., Cantù,S. Pilotti,S., Mariani,L., Gronchi,A., Henson,J.D., Reddel,R.R. and Zaffaroni,N. Presence and Clinical Relevance of Telomere Maintenance Mechanisms in Liposarcoma. Cancer Research (Manuscript submitted, 2006)

6. Jiang,W.Q., Zhong,Z.H., Henson,J.D. and Reddel,R.R. (2005). Screen of candidate genes required for ALT by RNA interference and methionine restriction. Oncogene (Manuscript submitted 2006)

7. Bowman,E.D., Mechanic,L., Henson,J.D., Reddel,R.R., Welsh,J., Khan,M., Hewitt,S., Dennis,P. and Harris.C.C. AKT phosphorylation and telomere lengthening in lung cancer prognosis (Manuscript in preparation).

Presentations

1. Henson,J.D., Yeager,T.R., Robinson,R.A., Yoo,J., Robinson,B.G., Learoyd,D.L., Jellinek,D.A., Wharton,S.B., Royds,J.A., Eeles,R. and Reddel,R.R. Investigating Alternative Lengthening of Telomeres (ALT) in human tumors by detection of ALT-Associated PML Bodies. Lorne Cancer Conference, 2002. (Winner of Poster Prize)

2. Henson,J.D., Yeager,T.R., Robinson,R.A., Yoo,J., Robinson,B.G., Learoyd,D.L., Jellinek,D.A., Wharton,S.B., Royds,J.A. and Reddel,R.R. Investigating Alternative Lengthening of Telomeres (ALT) in human tumors by detection of ALT-Associated PML Bodies. Australian Society for Medical Research, XIth NSW Scientific Meeting, 2002.

3. Henson,J.D., Yeager,T.R., Arbuckle,S.M., Robinson,R.A., Yoo,J., Jellinek,D.A., Wharton,S.B., Royds,J.A., McCarthy,S.W., Eeles,R. and Reddel,R.R. Investigating Alternative Lengthening of Telomeres (ALT) in human tumors by detection of ALT-Associated PML Bodies. Australian Telomere Workshop I, 2002.

4. Henson,J.D., Yeager,T.R., McCarthy,S.W., Pollock,R.E., Arbuckle,S.M., Robinson,R.A., Yoo,J., Robinson,B.G., Learoyd,D.L., Jellinek,D.A., Wharton,S.B., Royds,J.A., Eeles,R. and Reddel,R.R. Investigating Alternative Lengthening of Telomeres (ALT) in human tumors by detection of ALT-Associated PML Bodies (APBs). Lorne Cancer Conference, 2003.

5. Henson,J.D., Hannay,J.A., McCarthy,S.W., Royds,J.A., Yeager,T.R., Robinson,R.A., Wharton,S.B., Jellinek,D.A., Arbuckle,S.M., Yoo,J., Robinson,B.G., Learoyd,D.L., Stalley,P.D., Bonar,S.F., Yu,D., Pollock,R.E., and Reddel,R.R. A robust assay for alternative lengthening of telomeres (ALT) in tumors demonstrates the significance of ALT in sarcomas and astrocytomas. Australian Telomere Workshop II, 2004.

6. Henson,J.D., Hannay,J.A., Royds,J.A., McCarthy,S.W., Yeager,T.R., Nuemann,A.A., Robinson,R.A., Wharton,S.B., Jellinek,D.A., Arbuckle,S.M., Yoo,J., Harris,C.C., Robinson,B.G., Learoyd,D.L., Stalley,P.D., Bonar,S.F., Yu,D., Pollock,R.E., and Reddel,R.R. Development of an assay for Alternative Lengthening of Telomeres (ALT). Children's Hospital Westmead, Grand Rounds, 2004.

7. Henson,J.D., Yeager,T.R., McCarthy,S.W., Pollock,R.E., Arbuckle,S.M., Bonar,S.F., Robinson,R.A., Yoo,J., Jellinek,D.A., Wharton,S.B., Royds,J.A., Robinson,B.G., Learoyd,D.L., Eeles,R. and Reddel,R.R. A robust assay for alternative lengthening of telomeres (ALT) in tumors demonstrates the significance of ALT in sarcomas and astrocytomas. Australian Health and Medical Research Congress, 2004.

8. Henson,J.D., Hannay,J.A., McCarthy,S.W., Royds,J.A., Yeager,T.R., Robinson,R.A., Wharton,S.B., Jellinek,D.A., Arbuckle,S.M., Yoo,J., Robinson,B.G., Learoyd,D.L., Stalley,P.D., Bonar,S.F., Yu,D., Pollock,R.E., and Reddel,R.R. A robust assay for alternative lengthening of telomeres (ALT) in tumors demonstrates the significance of ALT in sarcomas and astrocytomas. Children's Cancer Institute Australia, Seminar, 2005.

9. Henson,J.D., Hannay,J.A., McCarthy,S.W., Royds,J.A., Yeager,T.R., Robinson,R.A., Wharton,S.B., Jellinek,D.A., Arbuckle,S.M., Yoo,J., Robinson,B.G., Learoyd,D.L., Stalley,P.D., Bonar,S.F., Yu,D., Pollock,R.E., and Reddel,R.R. A robust assay for alternative lengthening of telomeres (ALT) in tumors demonstrates the significance of ALT in sarcomas and astrocytomas. Australian Institute for Medical Scientists, National Scientific Meeting, 2005.

Summary

Activation of a telomere maintenance mechanism is a vital step in the development of most cancers and provides a target for the selective killing of cancer cells. Cancers can use either telomerase or Alternative Lengthening of Telomeres (ALT) to maintain their telomeres and inhibition of either telomere maintenance mechanism can cause cancer cells to undergo senescence or apoptosis. Although telomerase inhibitors are undergoing clinical trials, on commencing this study very little was known about the role of ALT in cancer, what proteins were involved in its mechanism and regulation and how it could be targeted clinically. The primary aim of this thesis was to develop an assay for ALT suitable for examining archived tumour specimens and to begin using it to examine the prevalence and clinical significance of ALT in cancer. This assay and gene expression analysis was also used to identify genes that are involved in or associated with the activation of the ALT mechanism, to contribute towards the overall goal of an ALT cancer therapy.

The ALT mechanism involves recombination mediated replication and ALT cells have a marked increase in a range of recombinational events specifically at their telomeres. Presumably, as a consequence of this the telomere lengths of ALT cells are very heterogeneous and on average long. This can be detected by terminal restriction fragment (TRF) Southern analysis, which has been used previously as the definitive test for ALT activity. However, TRF analysis requires intact genomic DNA and is unsuitable for tumour specimens which are commonly archived by paraffin embedding. Another hallmark of ALT is ALT-associated PML bodies (APBs) which are the subset of PML bodies that contain telomeric DNA. Work done in this study to

consolidate APBs as a hallmark of ALT, combined with published data, showed 29/31 ALT[+], 3/31 telomerase[+] and 0/10 mortal cell lines/strains are APB[+]. The three APB[+]/telomerase[+] cell lines identified here had an order of magnitude lower frequency of APB[+] nuclei than the ALT[+] cell lines. APBs may be functionally linked to the ALT mechanism and contain the recombination proteins that are thought to be involved in the ALT mechanism. This study, in collaboration with Dr W-Q Jiang, strengthened this functional link by demonstrating that loss of ALT activity (as determined by TRF analysis) coincided with the disruption of APBs.

The detection of APBs was developed into a robust assay for ALT in archived tumour specimens using a technique of combined immunofluorescence and telomere fluorescence *in situ* hybridisation. It was demonstrated that the APB assay concurred exactly with the standard assay for ALT (TRF analysis) in 60 tumours for which TRF analysis gave unequivocal results. The APB assay may be a more appropriate technique in the case of tumour specimen heterogeneity, which may explain why the APB assay was able to give definitive results when TRF analysis was equivocal. We demonstrated that intratumoral heterogeneity for ALT does exist and this could explain why about 3% of tumours in this study were APB[+] but with more than a ten-fold reduction in the frequency of APB[+] nuclei. This study also made the novel discovery of single stranded C-rich telomeric DNA inside APBs which potentially could be used to make the APB assay more suitable for routine pathology laboratory use.

The APB assay was used to show that ALT is a significant concern for oncology. ALT was utilised in approximately one quarter of glioblastoma multiforme (GBM),

one third of soft tissue sarcomas (STS) including three quarters of malignant fibrous histiocytomas (MFH), half of osteosarcomas and one tenth of non-small cell lung carcinomas (NSCLC). Furthermore, the patients with these ALT[+] tumours had poor survival; median survivals were 2 years for ALT[+] GBM, 4 years for ALT[+] STS including 3.5 years for ALT[+] MFH and 5 years for ALT[+] osteosarcoma. ALT[+] STS and osteosarcomas were also just as aggressive as their ALT[-] counterparts in terms of grade and patient outcome. ALT status was not found to be associated with response to chemotherapy in osteosarcomas or survival in STS. ALT was however, less prevalent in metastatic STS.

The APB assay was a prognostic indicator for GBM and was correlated with three fold increased median survival in GBM (although this survival was still poor). ALT was more common in lower grade astrocytomas (88% ALT[+]) than GBM (24% ALT[+]) and ALT[+] GBM had an identical median age at diagnosis to that reported for secondary GBM. It is discussed that these data indicate that ALT was indirectly associated with secondary GBM and is possibly an early event in its progression from lower grade astrocytoma. This is relevant because secondary GBM have distinct genetic alterations that may facilitate activation of the ALT mechanism.

Putative repressors of ALT could explain why this study found that ALT varied among the different STS subtypes. ALT was common in MFH (77%), leiomyosarcoma (62%) and liposarcoma (33%) but rare in rhabdomyosarcoma (6%) and synovial sarcoma (9%). ALT was not found in colorectal carcinoma (0/31) or thyroid papillary carcinoma (0/17) which have a high prevalence of telomerase

activity and a reduced need for a telomere maintenance mechanism (low cell turnover), respectively.

A yeast model of ALT predicts that one of the five human RecQ helicases may be required for ALT. Using the APB assay to test for the presence of ALT in tumours from patients with known mutations in either WRN or RECQL4 it was demonstrated that neither of these RecQ helicases is essential for ALT. Although p53 and mismatch repair (MMR) proteins have been suggested to be possible repressors of ALT, there was no apparent increase in the frequency of ALT in tumours from patients with a germline mutation in p53 codon 273 or in colorectal carcinomas that had microsatellite instability and thus MMR deficiency. Also contrary to being a repressor of ALT but consistent with its ability to interact with a protein involved in the ALT mechanism, the MMR protein MLH1, was demonstrated to be present in the APBs of an ALT[+] cell line.

To further test for genes that may be involved in the ALT mechanism or associated with its activation, RNA microarray was used to compare the gene expression of 12 ALT[+] with 12 matched telomerase[+] cell lines; 240 genes were identified that were significantly differentially expressed ($p<0.005$) between the ALT[+] and telomerase[+] cell lines. Only DRG2 and SFNX4 were significantly differentially expressed after adjusting for the estimated false positive rate. Overall, DRG2, MGMT and SATB1 were identified as most likely to be relevant to the ALT[+] tumours and Western analysis indicated that DRG2 and MGMT levels were down-regulated after activation of ALT and up-regulated after activation of telomerase, whereas SATB1 protein levels appeared to be up-regulated after immortalisation but to a higher degree

with activation of ALT compared to telomerase. Since lack of MGMT is known to be a determinant of temozolomide sensitivity in GBM, the possibility that ALT and the APB assay could be used to predict temozolomide sensitivity is discussed. The microarray data was consistent with MGMT expression being suppressed by EGF ($p < 0.05$), indicating that caution may be needed with combining EGFR inhibitors with temozolomide in ALT cancers. One ALT[+] cell line which did not express MGMT had TTAA sequence in its telomeres. This could possibly have resulted from mutations due to lack of MGMT expression and a possible role for MGMT in the ALT mechanism is discussed.

Further analysis of the microarray data identified two groups of co-regulated genes ($p < 5x10^{-5}$): CEBPA, TACC2, SFXN4, HNRPK and MGMT, and SIGIRR, LEF1, NSBP1 and SATB1. Two thirds of differentially expressed genes were down-regulated in ALT. Chromosomes 10 and 15 had a bias towards genes with lower expression in ALT while chromosomes 1, 4, 14 and X had a bias towards genes with higher expression levels in ALT.

This work has developed a robust assay for ALT in tumour specimens which was then used to show the significance of ALT in sarcomas, astrocytomas and NSCLC. It has also identified genes that could possibly be molecular targets for the treatment of ALT[+] cancers.

Abbreviations

ADP	adenosine diphosphate
AEBSF	4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride
ALT	Alternative Lengthening of Telomeres
APB	ALT-associated PML body
AT	ataxia telangiectasia
ATCC	American Type Culture Collection
BCNU	bis-(2-chloroethyl)-nitrosourea
bp	base pair
BS	Bloom syndrome
BSA	bovine serum albumin
BUR	base unpairing region
CF	cystic fibrosis
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate
CI	confidence interval
CO-FISH	chromosome orientation fluorescence *in situ* hybridization
cpm	counts per minute
CSL	Commonwealth Serum Laboratories
DEPC	diethyl pyrocarbonate
DABCO	1,4 Diazabicyclo(2.2.2)octane
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DMSO	dimethylsulphoxide
DMEM	Dulbecco's Modified Eagle Medium

DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleoside 5′-triphosphate
DSB	double strand break
DTT	dithiothreitol
ECTR	extra-chromosomal telomeric repeats
EDTA	Ethylenediaminetetraacetate
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’- tetraacetate
ExoI	Exonuclease I
ExoIII	Exonuclease III
FBS	foetal bovine serum
FISH	fluorescence *in situ* hybridisation
FITC	fluorescein isothiocyanate
GBM	glioblastoma multiforme
HBS	HEPES-Buffered Saline
HJ	Holliday junction
HMT	histone methyltransferases
HPV	Human Papilloma Virus
HR	homologous recombination
HRP	horseradish peroxidase
hTERT	human Telomerase Reverse Transcriptase
hTR	human Telomerase RNA
LB	lysis buffer
LFS	Li Fraumeni syndrome
LHC	Laboratory of Human Carcinogenesis

LHC-BM	Laboratory of Human Carcinogenesis basal medium
LNS	Lesch-Nyhan syndrome
MAR	matrix attachment region
MBN	Mung bean nuclease
MFH	malignant fibrous histiocytoma
MMR	mismatch repair
MOPS	3-(N-morpholino)propane-sulphonic acid
MRN	MRE11/ RAD50/ NBS1 complex
MSI	microsatellite instability
MSS	microsatellite stable
NCI-Frederick	National Cancer Institute at Frederick Division of Cancer Treatment Tumor Repository
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NIGMS	National Institute of General Medical Sciences Human Genetic Cell Repository
NP40	Nonidet P-40
NSCLC	non small cell lung carcinoma
O^6-AG	O^6-alkylguanine
O^6-MG	O^6-methylguanine
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PD	population doubling
TWEEN-20	polyoxyethylene-sorbitan monolaurate
PVP-40	polyvinylpyrrolidone 40

RNA	ribonucleic acid
RNAse	ribonuclease
RPMI	Roswell Park Memorial Institute
RT	room temperature
RTS	Rothmund-Thomson syndrome
SBTI	soy bean trypsin inhibitor
SCE	sister chromatid exchange
SDS	sodium dodecyl sulphate
SIR	semi interquartile range
SMC	structural maintenance of chromosome (protein)
SSC	sodium chloride-trisodium citrate buffer
STS	soft tissue sarcoma
SV40	Simian virus 40
TBE	tris-borate-EDTA buffer
TE	tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TMM	telomere maintenance mechanism
TNE	tris-sodium chloride-EDTA buffer
TRAP	Telomere Repeat Amplification Protocol
TRD	telomeric rapid deletion
TRF	terminal restriction fragment
TRIS	tris(hydroxymethyl)aminomethane
v/v	volume for volume
WHO	World Health Organisation
WS	Werner syndrome

w/v	weight for volume

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