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Collagen Expression in Fibroblasts with a Novel *LMNA* Mutation

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Abstract

Laminopathies are a group of genetic disorders caused by *LMNA* mutations; they include muscular dystrophies, lipodystrophies and progeroid syndromes. We identified a novel heterozygous *LMNA* mutation, L59R, in a patient with the general appearance of mandibuloacral dysplasia and progeroid features. Examination of the nuclei of dermal fibroblasts revealed the irregular morphology characteristic of *LMNA* mutant cells. The nuclear morphological abnormalities of *LMNA* mutant lymphoblastoid cell lines were less prominent compared to those of primary fibroblasts. Since it has been reported that progeroid features are associated with increased extracellular matrix in dermal tissues, we compared a subset of these components in fibroblast cultures from *LMNA* mutants with those of control fibroblasts. There was no evidence of intracellular accumulation or altered mobility of collagen chains, or altered conversion of procollagen to collagen, suggesting that skin fibroblast-mediated matrix production may not play a significant role in the pathogenesis of this particular laminopathy.

Keywords

Progeroid syndrome; Laminopathy; Collagen; Aging

Introduction

The *LMNA* gene encodes lamin A/C nuclear intermediate filaments [1] . Mutations at the *LMNA* locus are responsible for a number of disorders collectively referred to as laminopathies. Most laminopathies involve tissues of mesenchymal origins, resulting in such features as cardiac and/or skeletal muscular dystrophy, lipodystrophy and progeroid syndromes [2,3]. A small subset of patients exhibit neuropathies known as Charcot-Marie-Tooth disease type 2B1 [4].

There are two broad classes of *LMNA* mutations – missense mutations and splicing variants. Amino acid substitutions comprise the majority of disease-causing *LMNA* mutations [2]. These are located across the coding region and can result in Emery-Dreifuss muscular dystrophy (EDMD2, autosomal dominant, and EDMD3, autosomal recessive) [5], dilated cardiomyopathy (DCM1A)[6] , limb-girdle muscular dystrophy type 1B (LGMD1B) [7], Dunnigan-type familial partial lipodystrophy (FPLD2) [8], mandibuloacral dysplasia (MAD) [9], and atypical Werner syndrome [10]. A small number of truncation mutations are also

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reported in DCM and muscular dystrophies [2]. Overlapping features of these laminopathies are not unusual [11,12], and, in the case of DCM, include conduction defects, arrhythmias, and left ventricular dilations that can lead to heart failure [6,13,14]. The lipid disorders in patients with lipodystrophies are additional risk factors for cardiovascular disease.

Splicing mutations of exon 11 comprise the second type of *LMNA* mutations. This group of mutations results in the in-frame deletion and elimination of proteolytic cleavage sites at the C-teminus of lamin A. Cleavage by the ZMPSTE24 protein is required for the maturation of pre-lamin A to mature lamin A [15]. The most common mutation responsible for the Hutchinson-Gilford progeria syndrome (HGPS), G608G, is an exon 11 splice mutation that results in a fifty amino acid deletion [16,17]. Milder forms of HGPS are due to a different 35 amino acid deletion [18]. The most severe laminopathy, restrictive dermopathy, results in neonatal death. This disease is due to a splicing mutation that skips 90 amino acids of exon 11 [19]. Interestingly, ZMPSTE24 mutations are also reported in MAD and restrictive dermopathy [15,19]. The most striking cardiovascular manifestations of HGPS is a form of severe atherosclerosis. Patients usually die from myocardial infarction and/or congestive heart failure at a median age of 13.

The pathological changes in the arteries of individuals with HGPS have been described as being very similar to that of the atherosclerosis seen in the general population [20]. The arteries of HGPS patients, however, show particularly severe depletion of smooth muscle cells and derangement of basement membrane [21]. It might therefore be best described as an arterial smooth muscle dystrophy. Moreover, unlike common forms of atherosclerosis, type I collagen has been shown to be dramatically reduced in the arterial adventitia and media and in both external and internal basement membranes of HGPS patients. Additionally, type I collagen is overrepresented in the arterial intimas of these patients [22]. Type III collagen is distributed in all three layers of arterial wall, as seen in control arteries [22]. Microarray studies of HPGS fibroblasts have so far failed to elucidate the pathogenesis [23,24].

We recently identified a novel *LMNA* mutation in a 16 year-old female originally referred to our International Registry of Werner Syndrome (University of Washington, Seattle, WA) because of progeroid features and a differential diagnosis that included a collagen disorder. No mutations could be detected in the coding sequence or the promoter of the *WRN* gene, and Western analysis revealed that the levels of WRN protein were normal Abnormal nuclear morphology of cultured somatic cells was consistent with a laminopathy disorder. Given the clinical suspicion of a collagen disorder and the importance of mesenchmal pathology in progeroid syndromes, we examined type I and III collagen expression in fibroblast cultures from this patient and controls. The results failed to document altered matrix expression in cells from our patient.

Materials And Methods

Patients and samples

The patient was referred to the International Registry of Werner Syndrome (University of Washington, Seattle, WA) (http:www.wernersyndrome.org). A lymphoblasotid cell line (LCL) was established by transformation of B lymphocytes from a peripheral blood sample via a preparation of Epstein-Barr virus. Primary fibroblast cultures were established from a punch biopsy of skin of the medial aspect of the mid upper arm [25]. The protocols were approved by the Human Subjects Review Committee of the University of Washington, Seattle, WA.

Nucleotide sequencing

Genomic DNA was isolated from the LCL cells. Each exon of the LMNA gene was amplified and sequenced with an ABI PRIZM 310 (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction [10].

Cell culture and nuclear morphology

Primary fibroblasts were established via explants from the skin biopsy and maintained under standard tissue culture conditions (give these conditions, including media, additions to media, including amount and type of serum, CO2 and oxygen, pH, temp., humidity, conditions of passaging, including trypsin/versene, degree of confluence when passaged, etc., unless given in another publication) . Fractions of the dividing cells were determined by 3H-thymidine incorporation for 72hrs followed by autoradiography. The percentage of labeled nuclei (labeling index) was determined by counting a minimum of 200 cells [26]

For nuclear morphology, exponentially growing culture were plated on glass coverslips, fixed with 3% paraformaldhyde in PBS, pH 7.4, permealilized with 1% Triton-X in PBS, and mounted with DAPI (diaminophenylindole) on the coverglass. Nuclei were visualized with the Nikon Upright Microscope at the Keck Center for Imaging, University of Washington. Seattle, WA [10,26]. For the quantitation of nuclear irregularity, nuclear contour ratio was determined in minimum of a hundred randomly chosen nuclei with the MetaMorph program. Statistical significance was determined by the student t-test and f-test [26].

Assay of collagen production

Production of collagens in the culture media were determined by proline incorporation as previously described [27,28]. Primary fibroblasts were grown in the presence of ascorbic acid containing 1 μCi ^{14}C proline/ml for 20hrs. Media and cell layers were harvested separately, lyophilized and dissolved in 0.5M acetic acid. Samples were separated by 5% SDS-PAGE, and visualized by autoradiography. For the conversions of procollagens to collagens, the samples were digested with 50 μg/ml pepsin for 4 hr at 15°C and stopped by 0.5 μg/ml pepstatin.

Results

Case Report

Our patient is a 17 year-old Caucasian female of North European origin. No significant health issues were reported at birth or during her first 5 years of life. Around age 5, she developed recurrent shoulder dislocations and joint contractures, most noticeably of her small fingers. Osteoporosis was diagnosed at age 8, along with abnormal skin findings including teleangiectasias, sclerodactyly, and poikilodermia. She had a history of poor wound healing. On examination, she was 151cm tall (−2SD) and weighed 39kg (−2.5SD). Her facial features were unusual; there were small ears, a narrow nose and a very small chin. In addition to the above skin findings, soft tissue calcification on the left elbow was noted. She had a Levine II/VI pan systolic murmur. An echocardiogram revealed mild-to-moderate mitral regurgitation. She was later diagnosed with cardiomyopathy. Her vision was normal with no evidence of cataracts.

The patient was referred to the International Registry of Werner Syndrome with a possible diagnosis of Werner syndrome. Sequencing analysis of the *WRN* gene showed no mutation, and the WRN protein was of the expected size and expressed at a level comparable to those of controls. Studies were initiated to rule out atypical Werner syndrome and associated mutations in the *LMNA* gene.

A Novel LMNA mutation associated with the abnormal nuclear morphology

Sequencing of the *LMNA* gene revealed a heterozygous T-to-C alteration at c.176 (Fig. 1). This alteration was confirmed by sequencing in the reverse direction. This change is expected to result in the substitution of arginine for leucine at amino acid 59 (L59R) within the *LMNA* gene. Neither of the parents carried this mutation, indicating that this was a *de novo* mutation. This change was not seen in any of 116 controls from a population-based sample of elderly US residents, most of whom were of northern European origin (DNA bank, National Long-Term Care Survey (Department of Pathology, University of Washington, Seattle, WA, USA) [10], thus making it unlikely that this was a polymorphism.

One of the hallmarks of *LMNA* mutations is abnormal nuclear morphology. We therefore examined the nuclear morphology in the primary fibroblasts derived from this patient. Figure 2 shows typical confocal microscopic fields after DAPI staining. The labeling index of the control line, 82-6, was 88% and the index of our patient's (NEWFL1010) fibroblasts was 73%, indicating that both lines were at a relatively early passage. The nuclei of the *LMNA* mutant fibroblasts showed denting, blebbing and irregular margins compared to the control lines (Fig. 2).

The degree of the abnormality of nuclear morphology was calculated by the nuclear contour ratio, $(2\pi r/\text{perimeter})^2$, which would be 1 if the nucleus is completely round and less than 1 if it is not. These ratios were 0.724 (± 0.121) for NEWFL1010 compared to 0.842 (± 0.065) for 82-6, indicating that morphology of *LMNA* mutant nuclei were more irregular and varied compared to the control (t-test $P < 0.001$).

Variation of the nuclear contour ratio among LMNA mutant cell lines

In order to assess the significance of the alterations in nuclear shape, we examined other cell lines with *LMNA* mutations (Table 1). Since there is considerable variation of mean nuclear contour ratios among control fibroblasts lines [26], a comparison of the variances using f-tests is more suitable than a comparison of the averages using t-tests. Using f-tests, the nuclear contour ratio of primary fibroblasts from cells with the R133L mutation (PORTU8020) was 0.800 ± 0.152 whereas fibroblasts obtained from a wild type female sibling control, PORTU8010, was 0.815 ± 0.073 ($P<0.0001$). On the other hand, the nuclear contour ratio of another *LMNA* mutant cell line, ATLAN1010, an A57P mutation, was 0.841 ± 0.080, which was not statistically different from either of two control lines ($P = 0.220, 0.347$). These data indicate that the abnormal nuclear morphology, though characteristic of somatic cells from patients with mutations in *LMNA* and *LMNA*-related genes, is not always prominent in primary skin fibroblasts.

Examination of the nuclear morphology of LCLs revealed a similar trend. Comparison of the variances within the PORTU pedigree again revealed a significant difference between the patient and the sibling (0.765 ± 0.203 vs 0.768 ± 0.159, <0.0002). The variance was not significantly different between the ATLAN patient and sibling (0.799 ± 0.224 vs 0.722 ± 0.229, $P = 0.504$). The differences in the variances of patient and controls for the LCL pairs was less that those of primary fibroblasts for both the PORTU and ATLAN pedigrees.

Extracellular matrix expression in LMNA mutant fibroblasts

SDS-PAGE analysis of the medium showed that the levels of proline incorporation of the bands corresponding to the procollagen chains, α1(III), α1(I) and α2(I), in *LMNA* mutant fibroblasts were similar to those of the control fibroblasts (Fig. 3A). Following pepsin treatment, the patterns of collagens, α1(III), α1(I) and α2(I) showed no difference between *LMNA* mutant and control fibroblasts (Fig. 3B). Similarly, assays of cell lysates showed no evidence of intracellular accumulation, altered mobility of chains, or altered conversion of procollagen to

collagen (data not shown). These results indicate that the *LMNA* mutations we investigated do not quantitatively alter the synthesis of these extracellular matrix components at least in the primary skin fibroblasts.

Discussion

LMNA mutations have been identified in an increasing numbers of genetic conditions, including muscular dystrophies, lipodystrophies and progeroid syndromes. The international Registry of Werner Syndrome (University of Washington, Seattle, WA) has been receiving a spectrum of Werner-like patients for diagnosis. As a result, we have identified *LMNA* mutations in four patients with atypical Werner syndrome [10]. We report a novel *LMNA* mutation, L59R, in a fifth patient presenting with what we have operationally defined as an atypical Werner syndrome. Given the molecular findings, however, this subject might be more appropriately referred to atypical or novel laminopathy.

Identification of this *LMNA* mutation in association with the clinical phenotype of our patient suggests the diagnosis of a laminopathy, most likely an atypical form of MAD. The diagnosis of laminopathy is further supported by the demonstration of abnormal nuclear morphology. The variance, rather than average, of the nuclear contour ratio appears to be more suitable for the assessment of the degree of abnormality. However, such abnormalities are not always seen in *LMNA* mutant cells. Filesi et al [29] reported a MAD case with homozygous R527H mutations that showed minimal degree of nuclear blebbings accompanied by low level of pre-lamin A accumulation.

Because the causal relationship between *LMNA* mutations and a range of progeroid syndromes has only recently been established, *LMNA* mutants have not been extensively studied by biogerontologists. Reduced production of type I and III collagens has been described in primary fibroblasts derived from the aged skin [30,31]. These changes are associated with an increased expression of matrix metalloproteinase and a reduced expression of tissue inhibitor of metalloproteinases in skin samples from aged people [32]. Such findings were also reported in dermal fibroblasts that underwent senescence in culture [33]. An additional report describes how collagen production determined by H3-labeled proline incorporation is increased in fibroblasts obtained from *LMNA* null mice compared to mutant mice complemented with lamin A and C cDNA [34]. It is therefore if importance to investigate the dynamics of extracellular matrix in a variety of human laminopathies. The present study makes a beginning effort in that direction. The results show that primary skin fibroblasts with *LMNA* mutations produce collagen type I and III at levels similar to that of control fibroblasts.

The manifestations of aging are thought to be particularly pronounced in the extracelular matrix, the primary component of which is collagen. The characteristic change of aged extracellular matrix is the accumulation of collagen glycoxidation generated by the so-called Maillard reaction. These advanced glycation endproducts (AGEs) are seen in aged skin from a wide variety of mammalian species [35], and has been implicated in the apoptosis of dermal fibroblasts [36]. Our initial investigation of collagen protein production therefore needs to be expanded to include the examination of these biomarkers of aging. To this end, all biological materials described in this report will be made available to qualified researchers via the International Registry of Werner Syndrome (http://www.wernersyndrome.org).

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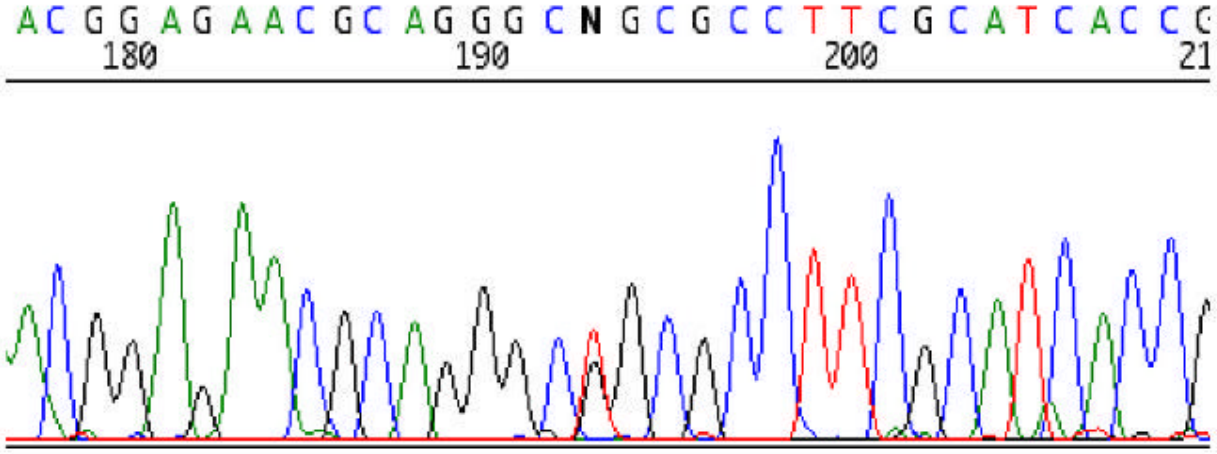

Figure 1.

Detection of the heterozygous *LMNA* mutation. The nucleotide N indicates the presence of heterozygous alteration resulting in L59R (CTG->CCG) mutation.

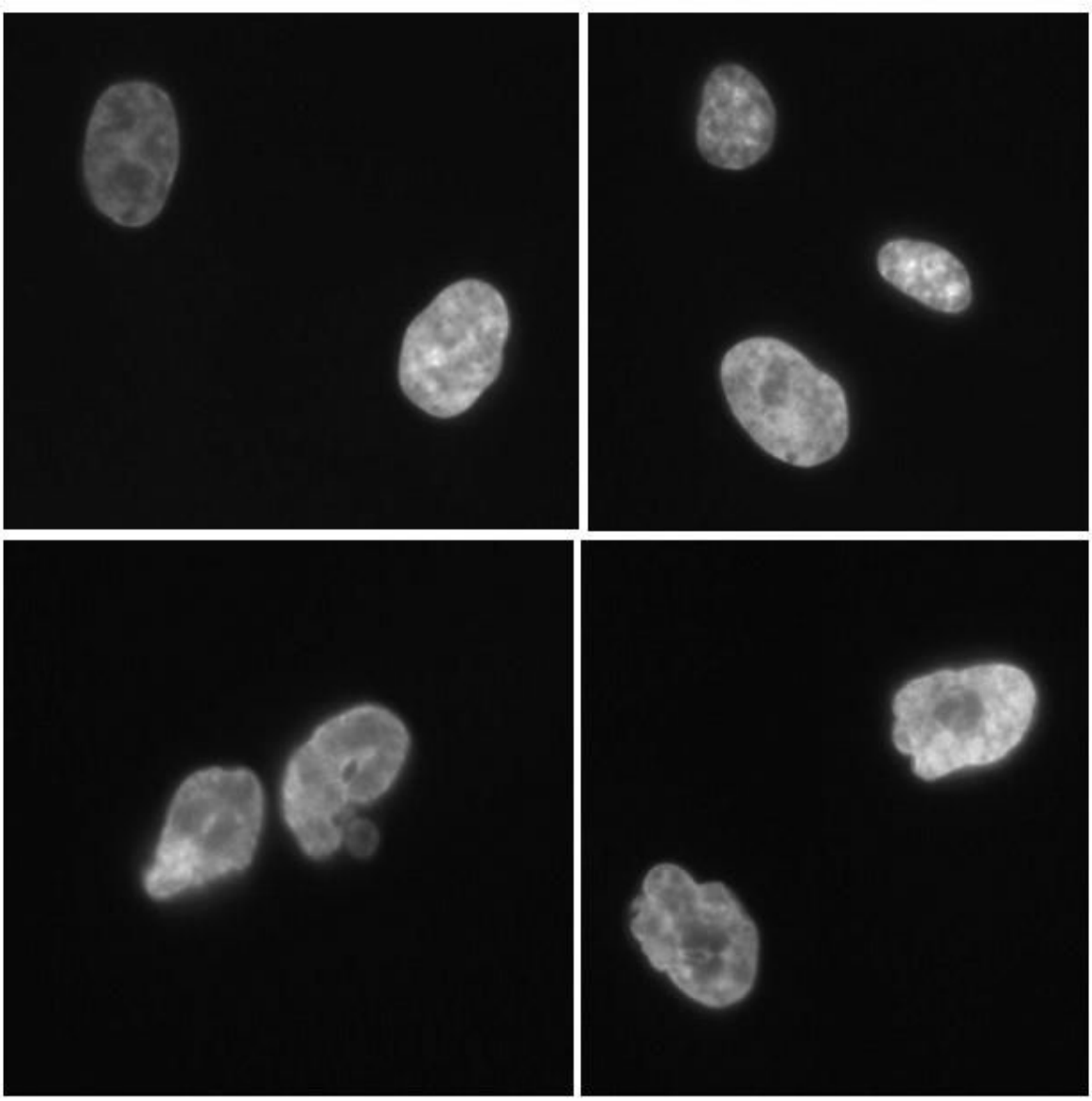

Figure 2.
Nuclear morphology of the *LMNA* mutant fibroblasts. Top two panels show the DAPI staining of the control fibroblasts, 82-6. Bottom two panels show that of NEWFL1010 fibroblasts.

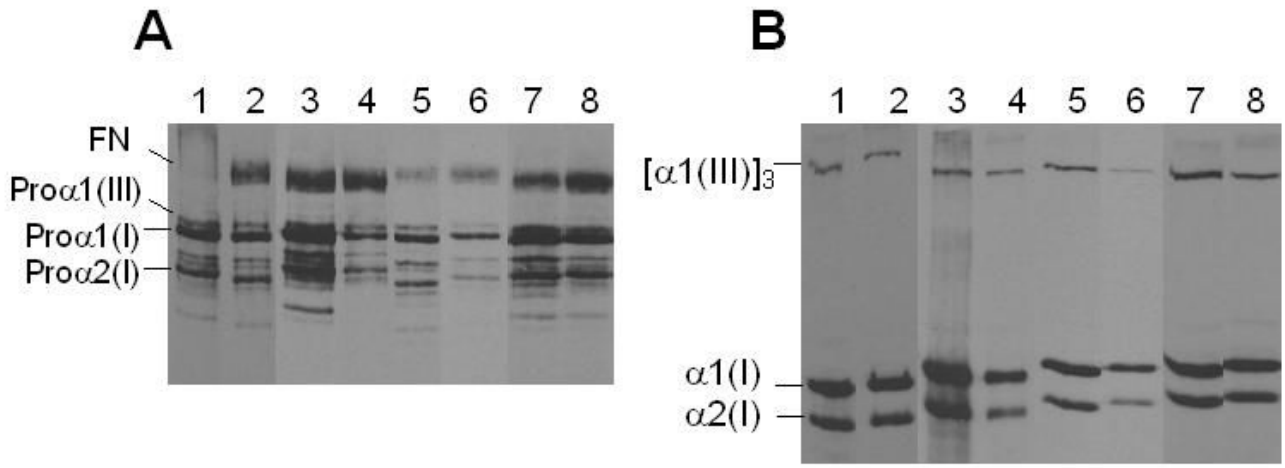

Figure 3.

Collagen production of the *LMNA* mutant fibroblasts. SDS-PAGE of radiolabeled procollagens (A) and collagens (B) from the culture media . Lane 1, 3, 5 and 7, control fibroblasts, A8; lane 2, ATLAN1010; lane 4, NEWFL1010; lane 6, PORTU8010; and lane 8 PORTU8020. All test sample data are paired with the control data used within the experiments.

Table 1

Nuclear contour ratios of the *LMNA* mutant fibroblasts and lymphoblastoid cell lines (LCL).

Registry number	LMNA Mutation	Labeling Index (%)	Nuclear Contour Ratio	P (f-test)
Primary fibroblasts				
NEWLF1010	L59R	73	0.724 ± 0.121	<0.002, <0.001*
PORTU8010	R133L	70	0.800 ± 0.152	<0.0001, <0.0001*
ATLAN1010	A57P	65	0.841 ± 0.080	0.220, 0.347*
PORTU8020	wt	81	0.815 ± 0.073	0.502**
82-6	wt	88	0.842 ± 0.065	NA
LCL				
PORTU8010	R133L	NA	0.765 ± 0.203	<0.0002
PORTU8020	wt	NA	0.768 ± 0.159	
ATLAN1010	A57P	NA	0.799 ± 0.224	0.504
ATLAN1020	wt	NA	0.722 ± 0.229	

* vs. 82-6 and vs. PORTU8020

** vs. 82-6